

NEUROHORMONAL PEPTIDES
IN GUT ENDOCRINE CELLS
AND RELATED TUMOURS

An immunocytochemical study

by

Jan Alumets

Lund 1981

NEUROHORMONAL PEPTIDES
IN GUT ENDOCRINE CELLS AND RELATED TUMOURS

An immunocytochemical study

by

Jan Alumets

Department of Histology, University of Lund, Lund, Sweden

Lund 1981

CONTENTS

INTRODUCTION

ASPECTS ON THE IMMUNOCYTOCHEMICAL PROCEDURE

ENDOCRINE CELLS OF THE GUT AND PANCREAS

Distribution and Ultrastructure

Glucagon

Secretin

Neurotensin

Somatostatin

Enkephalin

Substance P

Ontogeny

TUMOURS STORING NEUROHORMONAL PEPTIDES IN THE GUT,
PANCREAS AND OVARY

SUMMARY

ACKNOWLEDGEMENTS

REFERENCES



The present summary is based on the following papers, which in the text will be referred to by their Roman numerals:

- I. Sundler, F., Alumets, J., Holst, J., Larsson, L.-I. and Håkanson, R.: Ultrastructural identification of cells storing pancreatic-type glucagon in dog stomach. *Histochemistry* 50, 33-37, 1976
- II. Larsson, L.-I., Sundler, F., Alumets, J., Håkanson, R., Schaffalitzky de Muckadell, O.B. and Fahrenkrug, J.: Distribution, ontogeny and ultrastructure of the mammalian secretin cell. *Cell Tissue Res.* 181, 361-368, 1977
- III. Sundler, F., Håkanson, R., Hammer, R.A., Alumets, J., Carraway, R., Leeman, S.E. and Zimmerman, E.A.: Immunohistochemical localization of neurotensin in endocrine cells of the gut. *Cell Tissue Res.* 178, 313-321, 1977
- IV. Sundler, F., Alumets, J., Håkanson, R., Carraway, R. and Leeman, S.E.: Ultrastructure of the gut neurotensin cell. *Histochemistry* 53, 25-34, 1977
- V. Alumets, J., Sundler, F. and Håkanson, R.: Distribution, ontogeny and ultrastructure of somatostatin immunoreactive cells in the pancreas and gut. *Cell Tissue Res.* 185, 465-479, 1977
- VI. Alumets, J., Håkanson, R., Sundler, F. and Chang, K.-J.: Leu-enkephalin-like material in nerves and enterochromaffin cells in the gut. An immunohistochemical study. *Histochemistry* 56, 187-196, 1978
- VII. Sundler, F., Alumets, J. and Håkanson, R.: 5-Hydroxytryptamine-containing enterochromaffin cells: storage site of substance P. *Acta physiol. scand. Suppl.* 452, 121-123, 1977
- VIII. Alumets, J., Håkanson, R. and Sundler, F.: Ontogeny of endocrine cells in porcine gut and pancreas. An immunocytochemical study. Submitted to *Gastroenterology*.
- IX. Alumets, J., Sundler, F., Falkmer, S., Håkanson, R., Ljungberg, O., Mårtensson, H. and Nobin, A.: Neurohormonal peptides in endocrine tumors of the pancreas, stomach, and upper small intestine. An immunohistochemical study of 27 cases. Submitted to *Am.J.Surg. Pathol.*

- X. Alumets, J., Håkanson, R., Ingemansson, S. and Sundler, F.: Substance P and 5-HT in granules isolated from an intestinal argentaffin carcinoid. *Histochemistry* 52, 217-222, 1977
- XI. Alumets, J., Alm, P., Falkmer, S., Håkanson, R., Ljungberg, O., Mårtensson, H., Sundler, F. and Tibblin, S.: Immunohistochemical evidence of peptide hormones in endocrine tumors of the rectum. *Cancer* 1981 (accepted for publication)
- XII. Sporrang, B., Falkmer, S., Robboy, S.J., Alumets, J., Håkanson, R., Ljungberg, O. and Sundler, F.: Neurohormonal peptides in ovarian carcinoids. An immunohistochemical study of eighty-one primary carcinoids and of intra-ovarian metastases from six mid-gut carcinoids. *Cancer* 1981 (accepted for publication)
- XIII. Sporrang, B., Alumets, J., Clase, L., Falkmer, S., Håkanson, R., Ljungberg, O. and Sundler, F.: Neurohormonal peptide immunoreactive cells in mucinous cystadenomas and cystadenocarcinomas of the ovary. *Virchows Arch. A Path. Anat. Hist.* 1981 (accepted for publication)

INTRODUCTION

In 1902 Bayliss and Starling made the remarkable observation that intravenous injection of an extract of duodenal and jejunal mucosa stimulated secretion of pancreatic juice from the denervated pancreas. The active agent was named secretin. The observation marks the introduction of a new concept, according to which bodily functions are regulated not only by nerves but also by blood borne messengers (hormones). During the following half-century a whole series of gut hormones was described: among them gastrin (Edkins, 1905), cholecystikinin (CCK) (Ivy and Oldberg, 1928), substance P (Euler and Gaddum, 1931) and pancreozymin (Harper and Raper, 1943). After a period of slow progress during the forties and fifties this field has recently attracted renewed interest (Fig. 1). The introduction of various new analytical techniques has opened the doors to previously inaccessible information and during the last decade a great number of new peptide hormones and hormone candidates have been discovered, isolated and characterized.

Studies of the functional significance of the gut endocrine cell system are hampered by its special anatomical features. The endocrine cells are disseminated throughout the gut, and the various endocrine cell types overlap in their distribution and occur intermingled with other epithelial cell types. Hormones are normally present in small amounts in the gut making their isolation difficult. Progress in the field of peptide isolation has paved the way for the recent advance in knowledge. Amino acid sequencing techniques have disclosed the structure of many of these peptides, thereby making their synthesis possible. Thus, Gregory and Tracy (1964) described the amino acid sequence of gastrin. Later, the sequences of e.g. secretin (Mutt et al., 1970), cholecystikinin (Mutt and Jorpes, 1971), substance P (Chang and Leeman, 1970), gastric inhibitory peptide (GIP) (Brown and Dryburgh, 1971), motilin (Brown et al., 1973) and vasoactive intestinal peptide (VIP) (Mutt and Said, 1974) were revealed. Also the amino acid sequences of insulin (Sanger and Thompson, 1953), glucagon (Bromer et al., 1957), somatostatin (Brazeau et al., 1973) and pancreatic polypeptide (PP) (Kimmel et al., 1968; cf. Chance et al., 1979) were established. Radioimmunochemical methods for the quantitation of peptide hormones became available and their cells of origin were identified by immunocytochemistry. These methodological advances enabled studies directed at elucidating the functional

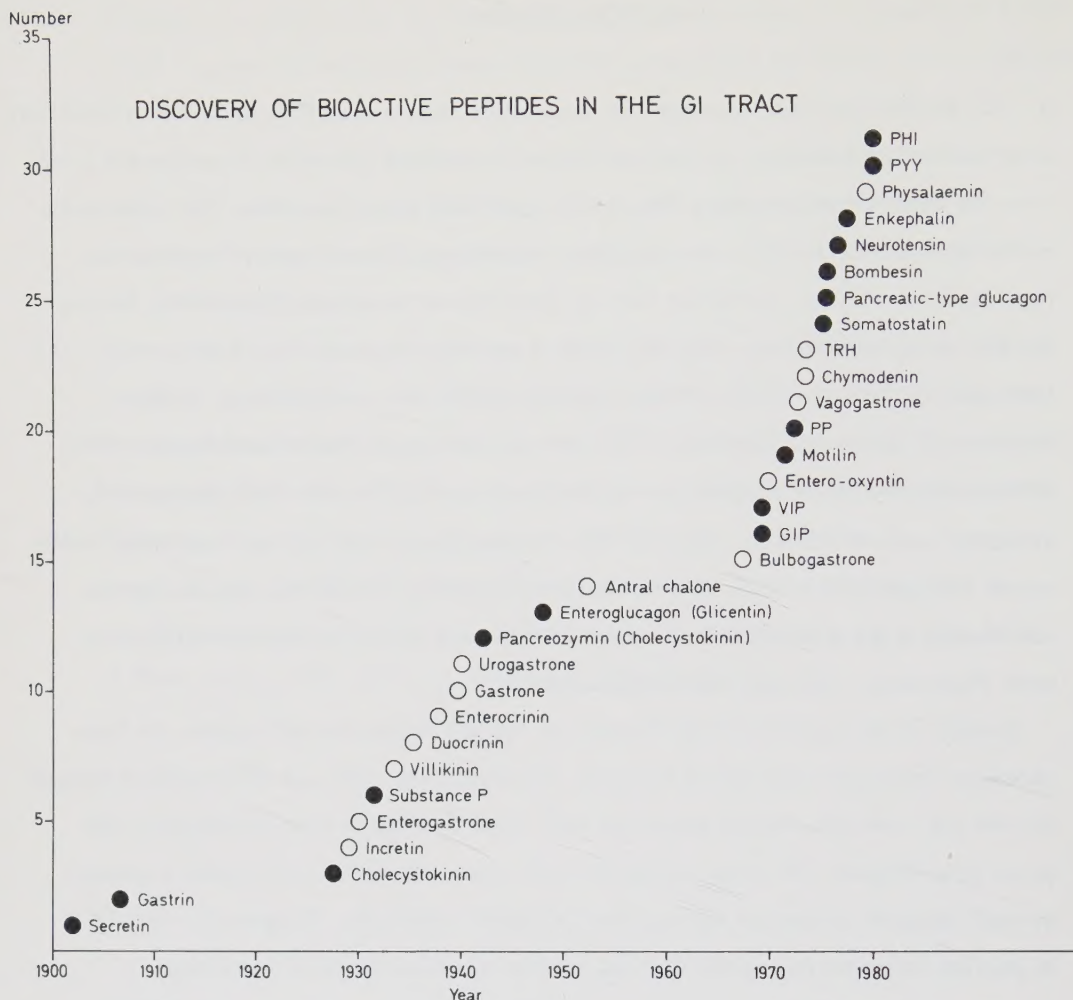


Fig. 1. Time of discovery of various bioactive peptides in the digestive tract. Filled circles denote established and sequenced neurohormonal peptides; open circles denote suggested bioactive but not yet identified peptides.

roles of the various gut hormones. The insulin and glucagon cells of the pancreatic islets were among the first cell types to be identified, soon followed by the gastrin and secretin cells. Subsequently, a great number of pancreatic and gut peptides have been localized immunocytochemically (cf. Creutzfeldt, 1980a).

The endocrine cells in the antrum of the stomach and the intestines are flask-

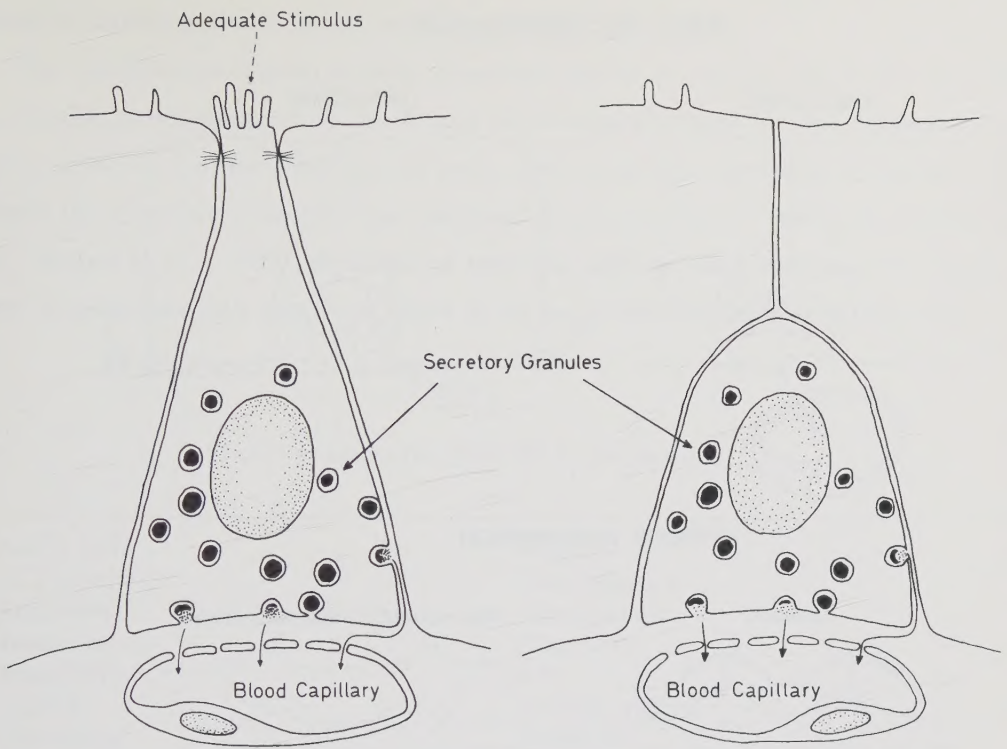


Fig. 2. Endocrine cells of open (left) and closed (right) type. Open cells reach the lumen via an apical process equipped with microvilli, whereas closed cells do not reach the lumen. Secretory products are released by exocytosis.

-shaped with a broad base and an apical process reaching the lumen (open type) (Fig. 2). The surface of the apical process is endowed with microvilli, probably provided with receptors responding to stimuli from the lumen. In the oxyntic gland area of the stomach the endocrine cells do not seem to reach the lumen (closed type). The reason for this is not known. Conceivably, these latter cells are stimulated by other mechanisms than are those of the open type.

The basal part of the cell is usually rich in secretory granules (Fig. 2), which represent the storage site of the peptide hormone. The granules are thought to deliver their content to the extracellular space by exocytosis and the secretory products reach the blood vessels beneath the basal membrane. The hormones are then transported by the blood to their targets (Fig. 3).

Certain endocrine-like cells, e.g. the somatostatin cells in the stomach are equipped

MODES OF COMMUNICATION

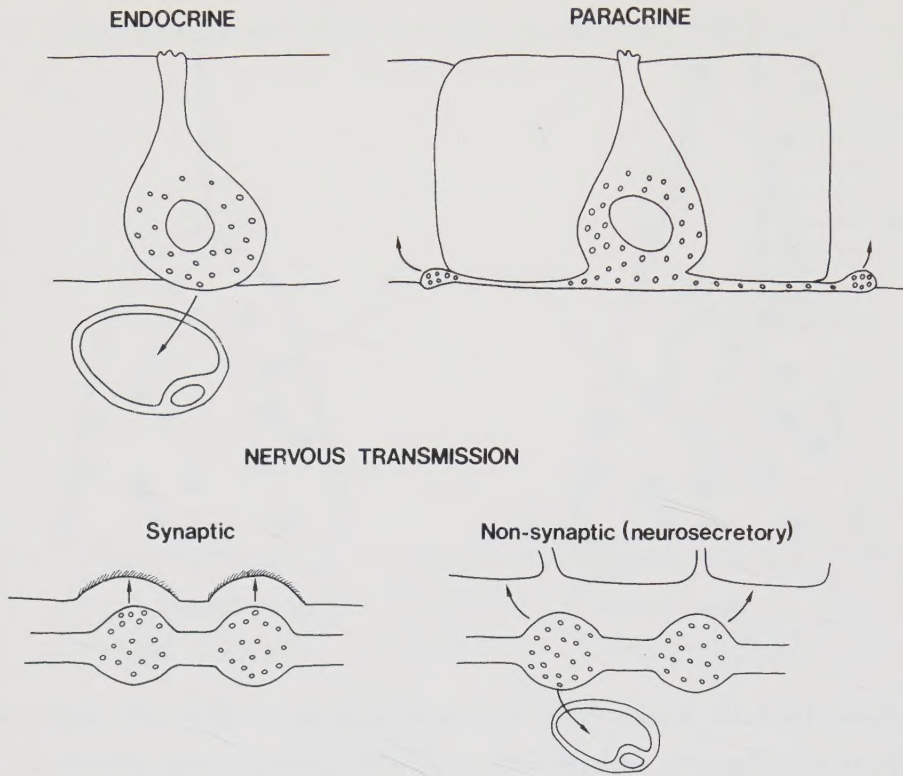


Fig. 3. The neuroendocrine system comprises endocrine/paracrine cells and neurones, all of which seem to employ amines and/or peptides as messenger compounds. The content of the granules of the endocrine cell reaches the blood vessels via exocytosis and is transported by the blood to the target organ. The paracrine cells give off long slender processes often ending in club-like swellings in close contact with their target cells. The messenger compounds are thought to be released in the immediate vicinity of the target cell. Nervous transmission occurs either by direct synaptic contact or by non-synaptic mechanisms.

with long slender processes at the base of the cell. These processes end in club-like swellings abutting on neighbouring cells, possibly representing target cells (Alumets et al., 1979a; Kusumoto et al., 1979; Larsson et al., 1979). Such a paracrine mode of communication is reminiscent of neural transmission in that the anatomical design

seems to facilitate direct cell-to-cell contact (Fig. 3).

The CNS harbours a great number of peptides (Table I), many of which are claimed to function as neurotransmitters or to have modulating effects on nervous transmission. Many peptides, first isolated from the brain, have later also been found in the gut (Table II). Conversely, several "gut peptides" have been found in the brain (Table II) (cf. Hökfelt et al., 1980). A number of these gut peptides have been found to occur both in endocrine cells and nerve fibers of the gut (Table III) (Sundler et al., 1978).

Table I

Known or suspected peptides in the brain

ACTH 1-39	α -MSH
ACTH 4-10	Neurophysins
Angiotensin	Neurotensin
Bombesin	Oxytocin
Bradykinin	PP
CCK-8	pro- δ -MSH
Endorphins	Prolactin
Enkephalins	Secretin
Gastrin-17, gastrin-4	Sleep-inducing peptide
GH	Somatostatin
Glucagon (gut-type)	Substance P
Insulin	TRH
LHRH	TSH
β -Lipotropin	Vasopressin
MIF	VIP

Table II

Brain peptides in the GEP region GEP peptides in the brain

Enkephalin	Bombesin
Neurotensin	CCK-8
Somatostatin	Gastrin-17, gastrin-4
Substance P	Glucagon (gut-type)
	VIP
	Insulin ?
	PP ?
	Secretin?

Table III

Peptides with a dual localization to endocrine
cells and neurons in the GEP region

Bombesin	Gastrin-4 ?
CCK-8	Neurotensin ?
Enkephalin	PP ?
Somatostatin	VIP ?
Substance P	

Gastrin-4 has been suggested to occur but has not been unequivocally identified. Neurotensin containing nerve fibers have been demonstrated in few mammals only. VIP occurs in both endocrine cells and neurons in lower vertebrates. In mammals VIP seems to be a neuronal constituent only. The identity of neuronal "PP" remains to be established.

Generally, the hormones of the gut show very little variation in the amino acid sequence between species. There are, however, exceptions: avian PP, for instance, differs greatly from mammalian PP variants (cf. Chance et al., 1979).

Some neurohormonal peptides display sequence homologies. Structurally related peptides have been grouped together in families. Thus, the gastrin family includes gastrin, CCK and caerulein; another peptide family is represented by secretin, glucagon, VIP and GIP (Fig. 4). Recent studies have revealed that many peptide hormones exist in more than one molecular form; e.g. gastrin has been found to occur in several varieties (cf. Rehfeld, 1979).

Although endocrine tumours of the gut and pancreas are rare, they have attracted much interest. This is so because some of them give rise to spectacular and severe symptoms, and because they offer a possibility to gain insight into the bioactivity profiles of the neurohormonal peptides. In addition, gut endocrine tumours provide opportunities for the isolation of large amounts of peptides including the hormones and their precursors (cf. Steiner, 1981).

The nomenclature used in the classification of endocrine tumours of the gut and pancreas is confusing. The widely used term carcinoid is sometimes used in a restricted sense to designate gut endocrine tumours storing 5-hydroxytryptamine (5-HT) and being argentaffin. The growth pattern displayed by these latter tumours is shared by virtually all

Secretin	1	His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Gln-Gly-Leu-Val-NH ₂	20
Glucagon		<u>His-Ser-Gln-Gly-Gly-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asp-Thr-</u>	
VIP		<u>His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂</u>	
GIP		<u>Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-Arg-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln</u>	
Gastrin	1	5 Glp-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂	15
CKK		20 -Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH ₂	
Caerulein		<u>SO₃H</u> Pyr-Gln-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-NH ₂	
Bombesin	1	5 Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH ₂	10
Eledoisin		<u>Glp-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH₂</u>	
Physalaemin		<u>Glp-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH₂</u>	
Substance P		<u>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂</u>	
Met-enkephalin	1	5 Tyr-Gly-Gly-Phe-Met	31
Leu-enkephalin		<u>Tyr-Gly-Gly-Phe-Leu</u>	
β -endorphin		<u>Tyr-Gly-Gly-Phe-Met-----Gln</u>	

Fig. 4. Amino acid sequences of the secretin family of peptides (secretin, glucagon, VIP and GIP), the gastrin family (gastrin, CCK and caerulein), the bombesin/substance P family (bombesin, eledoisin, physalaemin and substance P). At the bottom of fig. is shown the sequences of the enkephalins and β -endorphin. Residues common to two or more of the peptides are underlined.

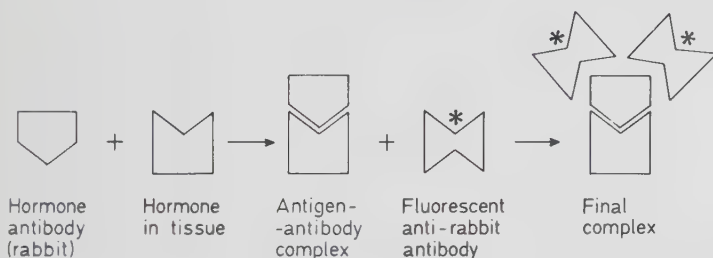
gastro-entero-pancreatic (GEP) endocrine tumours (cf. Jouanneau and Malafosse, 1971). Thus, the term carcinoid is often used in a broad sense to mean gut endocrine tumours in general (Soga, 1974; Dawson, 1976). To avoid confusion we prefer the term "endocrine tumour" over "carcinoid".

As mentioned above, GEP endocrine tumours may give rise to specific constellations of symptoms, e.g. the Zollinger-Ellison syndrome (cf. Zollinger et al., 1980) and the WDHA syndrome (cf. Morrison, 1980). The symptoms associated with these two syndromes are caused by gastrin and VIP, respectively. Immunochemical and immunocytochemical studies have shown most GEP endocrine tumours to be multihormonal, i.e. containing more than one peptide hormone producing cell type (Larsson et al., 1975a; Sundler et al., 1979; Creutzfeldt, 1980b). As a rule, however, only one of the peptides gives rise to the symptoms. Many tumours that produce neurohormonal peptides are clinically "silent" in that the symptoms are non-existent or vague and uncharacteristic. Consequently, they are often found incidentally.

ASPECTS ON THE IMMUNOCYTOCHEMICAL PROCEDURE

The indirect immunofluorescence method of Coons et al. (1955) or the peroxidase-antiperoxidase (PAP) procedure of Sternberger (1974) are in common use. In the indirect immunofluorescence technique (Fig. 5) the sections are exposed to the specific peptide antiserum in the appropriate dilution for various length of time (usually 3 hrs) at room temperature. After thorough rinsing in phosphate buffer they are incubated in fluorescein-labelled anti-rabbit (or anti-guinea-pig) IgG (for 30 min at room temperature). After another rinsing in phosphate buffer the sections are mounted in phosphate buffered glycerin and examined with a fluorescence microscope equipped with filters selected to give peak excitation at 490 nm.

IMMUNOFLUORESCENCE TECHNIQUE



IMMUNOPEROXIDASE (PAP) TECHNIQUE

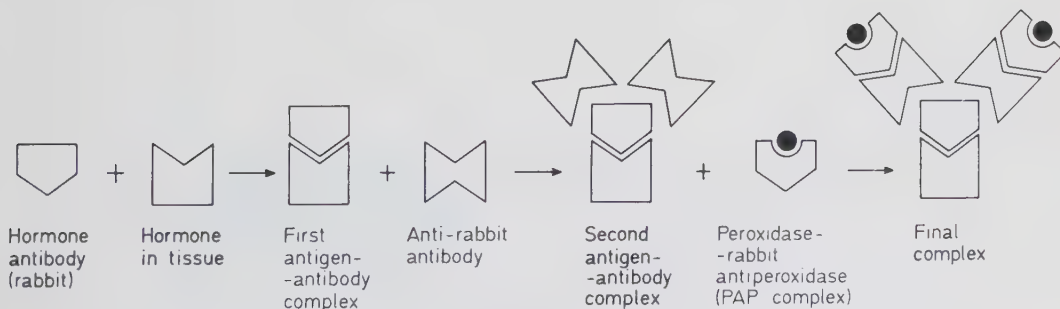


Fig. 5. Summary of the steps in the indirect immunofluorescence technique (top) and the immunoperoxidase (PAP) technique (bottom).

In the PAP procedure (Fig. 5) the sections are exposed to the peptide antiserum overnight at $+4^{\circ}\text{C}$. After rinsing in phosphate buffer the sections are incubated in unlabelled goat (or swine or sheep) anti-rabbit or anti-guinea-pig IgG for 30 min at room temperature followed by incubation for 30 min with PAP complex, i.e. peroxidase coupled to a rabbit (or guinea-pig) antibody raised against horseradish peroxidase. This is followed by incubation with a solution of 0.05% diaminobenzidine hydrochloride and 0.01% hydrogen peroxide in Tris buffer (pH 7.6) for 1 h. The sections are mounted in Permount^R and examined with a light microscope.

All peptide antisera contain 0.25% human serum albumin, and all solutions contain 0.25% Triton X-100 to facilitate penetration in the tissue. Sections from formalin fixed material are rinsed in phosphate buffer overnight prior to the immunocytochemical processing.

Endocrine cells can be identified ultrastructurally, using either the consecutive semithin/ultrathin section technique (Fig. 6) or direct PAP staining of ultrathin sections. For this purpose specimens are fixed in a mixture of 1% glutaraldehyde and 3% formaldehyde in 0.075 M phosphate buffer, pH 7.2, and postfixed in 1% osmium tetroxide. In

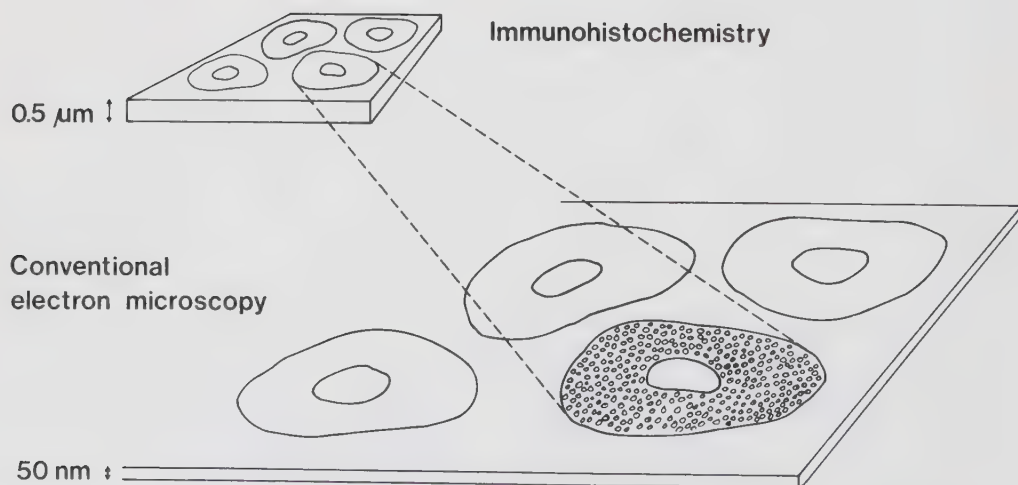


Fig. 6. The consecutive semithin/ultrathin section technique. The semithin section ($0.5\ \mu\text{m}$) is processed for immunohistochemistry (above); the immunoreactive cells in the semithin section are identified in the consecutive ultrathin section.

the first technique semithin ($< 0.5 \mu\text{m}$) sections are etched for 10 min with sodium methoxide according to Mayor et al. (1961) and osmium deposits removed with 1% periodic acid (applied for 10 min) (cf. Beauvillain et al., 1975), before the immunocytochemical staining (see above) The immunoreactive cells in the semithin section are identified in the adjacent ultrathin section (400–500Å) which has been contrasted with uranyl acetate and lead citrate. This allows a detailed evaluation of the fine structure of the immunoreactive cells. The major disadvantage of this technique is the difficulties involved in recognizing the immunoreactive cells unequivocally in the ultrathin section. Moreover, this technique does not identify the subcellular storage site of the immunoreactive material.

Immunostaining of ultrathin sections is performed in the following way: Ultrathin sections are placed on nickel or gold grids. After etching with 10% H_2O_2 for 20 min and removal of osmium deposits with 1% periodic acid for 5 min during stirring, the sections are incubated in normal sheep serum (in dilution 1:20) for 10 min. After rinsing in phosphate buffer the sections are incubated with the peptide antiserum for 3–4 hrs at room temperature. This is followed by incubation with unlabelled goat anti-rabbit or anti-guinea-pig IgG for 10 min, rinsing in phosphate buffer and incubation with PAP complex for 10 min. After rinsing in 0.05 M Tris buffer (pH 7.6) the sections are incubated in a solution of 0.05% diaminobenzidine hydrochloride and 0.01% hydrogen peroxide in Tris buffer (pH 7.6) during stirring for 5 min. After a short rinsing in re-distilled water the grids are incubated in a solution of 4% osmium tetroxide for 25 min and again rinsed in redistilled water. This technique can be used to identify the subcellular storage site of the immunoreactive material. One drawback, however, is that the immunoprecipitate often masks fine structural details. Also, the contrast of the sections is poor due to the removal of osmium.

Distribution and ultrastructure

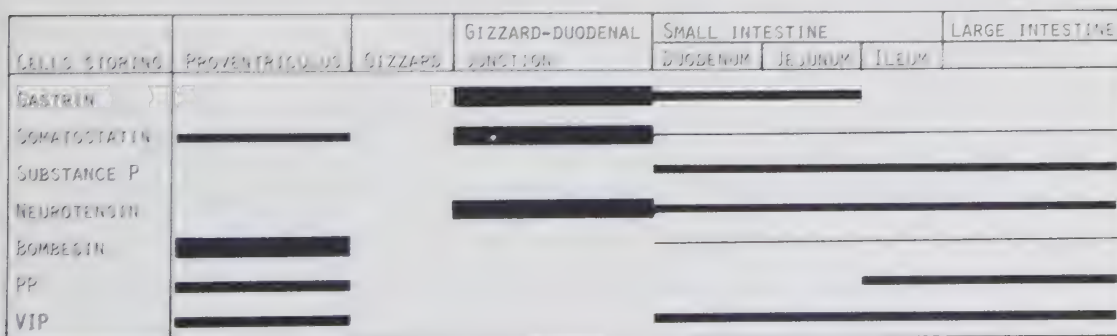
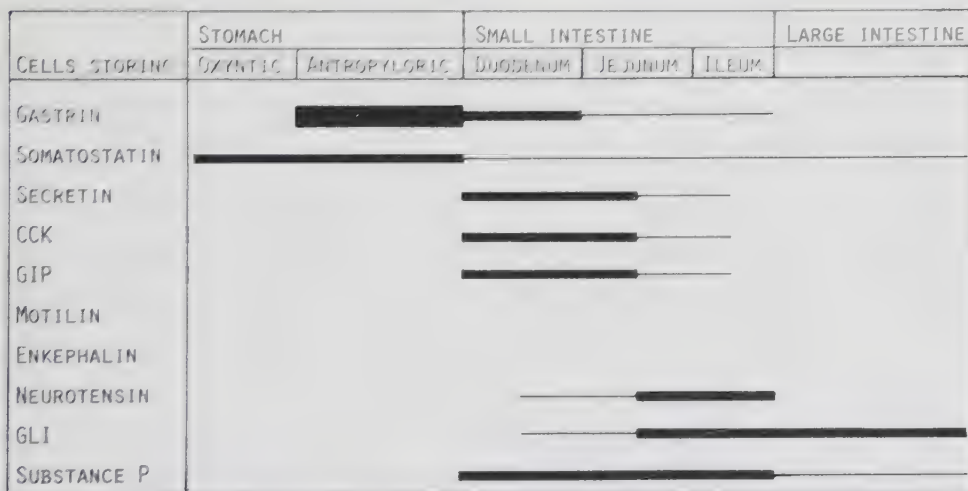
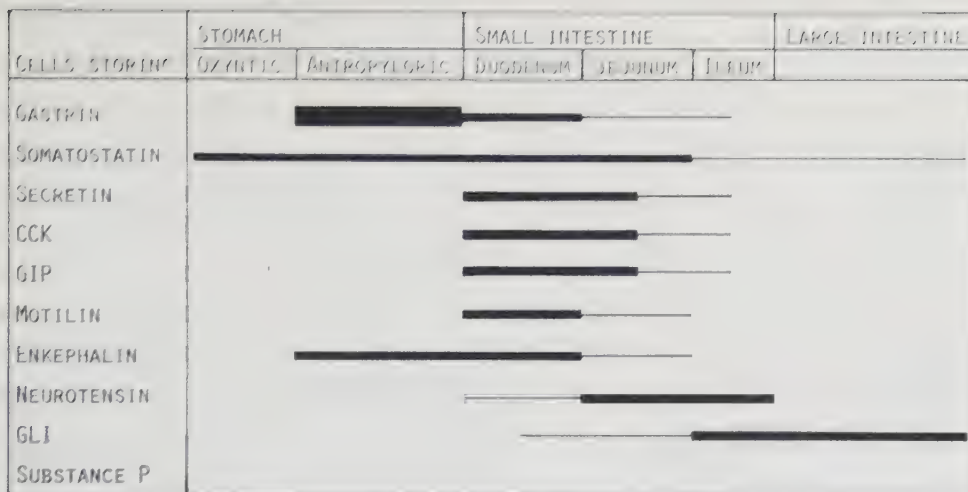
The general distribution of various endocrine cell types in the gut of several animals is illustrated in Fig. 7. The following deals primarily with some of the cell types extensively studied in our own laboratory. The insulin cells, PP cells, gastrin cells, CCK cells, GIP cells, and motilin cells will not be discussed here.

Glucagon

Glucagon was discovered as a hyperglycemic agent in the pancreas (Murlin et al., 1923; cf. Unger and Orci, 1976). Pancreatic glucagon is a 29 amino acid peptide, localized by immunocytochemistry to the A cells of the pancreatic islets (Baum et al., 1962). In most mammals the glucagon cells are located in the periphery of the islets. In certain species including man, glucagon cells also occur scattered within the central core of the islets.

A "glucagon-like" glycogenolytic and hyperglycaemic agent was demonstrated in extracts of the stomach and gut by Sutherland and de Duve in 1948 and the existence of immunoreactive glucagon in such extracts was described by Unger et al. (1961). In most species the concentration of immunoreactive glucagon is low in the stomach and duodenum but fairly high in the lower small intestine and the colon (cf. Holst, 1977). In the cat and dog, however, there are considerable amounts of immunoreactive glucagon in the oxyntic gland area (Unger et al., 1966). Recently, one variant of gut glucagon was isolated and named glicentin (Sundby et al., 1976). Sequence analysis revealed that glicentin, consisting of 100 amino acids, contains the glucagon 1-29 sequence (Jacobsen et al., 1977; cf. Conlon, 1980; Holst, 1980).

Using glucagon antisera reacting specifically with pancreatic-type glucagon and antisera cross-reacting with gut-type glucagon, it was possible to show that endocrine cells in the oxyntic gland area in cat and dog store pancreatic-type glucagon whereas those of the distal small intestine and large intestine harbour gut-type glucagon (Larsson et al., 1975c). Certain endocrine cells in the gastro-intestinal mucosa contain secretory granules very similar to those of the pancreatic glucagon cells (Orci et al., 1968; Forssman et al., 1969). In fact, the oxyntic gland area of some mammals, notable the dog, harbours cells indistinguishable from the pancreatic glucagon cells at



NUMEROUS CELLS MODERATE NUMBER OF CELLS RARE CELLS

Fig. 7. Distribution of endocrine cells in various gut segments of the pig (top), rat (middle) and chicken (bottom).

the ultrastructural level (Vasallo et al., 1969; Kubes et al., 1974; Sasaki et al., 1975; Solcia et al., 1975). The distribution and tinctorial properties of glucagon cells in the gut and the concentrations and properties of glucagon-like material in the gastro-intestinal tract suggested that the pancreatic-type glucagon in the stomach is produced in the A cells, whereas gut-type glucagon (glicentin) in the intestines is stored in L cells (Polak et al., 1971a, b; Baetens et al., 1976).

The existence of pancreatic-type glucagon in the A cells of the stomach of the dog was established by the consecutive semithin-ultrathin section technique (Grimelius et al., 1976; Paper I). The cells contain numerous round, membrane-bound cytoplasmic granules with uniformly high electron density indistinguishable from the granules in the pancreatic A cells. The dense core of the granules is separated from the limiting membrane by an electron-lucent halo (Fig. 8). The cells which are rather numerous, are of the closed type, i.e. they have no luminal contact. The L cell of the intestines was identified as the cell storing gut-type glucagon (Baetens et al., 1976; Grimelius et al., 1976).

Recently, immunoreactive glicentin was found to occur not only in the L cells of the distal small intestine and the colon, but also in the A cells of the pancreas and in the A cells of the dog stomach (Ravazzola et al., 1979; Ravazzola and Orci, 1980). Using the protein A-gold technique, which provides more ultrastructural details than the PAP technique, Ravazzola and Orci (1980) described the segregation of the glucagon and glicentin immunoreactive material in the secretory granules of the pancreatic glucagon cells. Whereas glucagon was localized to the dense core of the granules, glicentin seems to exist in the peripheral electron lucent zone.

Both immunochemical (Conlon et al., 1979; Tager et al., 1980) and immunocytochemical (Lorén et al., 1979; Tager et al., 1980) studies have demonstrated immunoreactive glucagon, probably gut-type glucagon, in a population of nerve fibers in the brain, thus adding this peptide to the already long list of peptides common to brain and gut.

Secretin

Although the existence of secretin was recognized already in 1902 (see Introduction), it was isolated only fairly recently (Jorpes and Mutt, 1961). The amino acid sequence was established a few years later (Mutt et al., 1965, 1970), making its synthesis

Cells storing	Species	Localization. Mean granule profile diameter (nm) $\pm$ S.D. (n)	Ultrastructure of the secretion granules
<u>Glucagon</u>	Dog	Stomach 310 ± 57 (343)	
<u>Secretin</u>	Cat	Duodenum 180 ± 55 (1145)	
	Pig	185 ± 52 (271)	
<u>Neurotensin</u>	Dog	Ileum 260 ± 64 (890)	
	Man	290 ± 76 (107)	
<u>Somatostatin</u>	Rat	Pancreas 170 ± 40 (548)	
	Cat	245 ± 57 (583)	
	Man	235 ± 45 (833)	
		Stomach 155 ± 39 (569)	
		265 ± 64 (813)	
		240 ± 48 (418)	
<u>Enkephalin</u>	Pig	Antrum 350 ± 90 (660)	
<u>Substance P</u>	Man	Ileal Carcinoid 180 ± 46 (226)	

Fig. 8. Mean granule profile diameter in various endocrine cells of different species together with schematic drawing of the granule ultrastructure.

possible (Bodanszky et al., 1967). The availability of antisera against porcine secretin made it possible to develop sensitive radioimmunoassays (Schaffalitzky de Muckadell and Fahrenkrug, 1976; cf. Schaffalitzky de Muckadell, 1980) and to localize the peptide by immunocytochemistry (Bussolati et al., 1971; Polak et al., 1971b).

In mammals, secretin cells predominate in duodenum and jejunum. They are rarely seen in the ileum (Paper II; cf. Schaffalitzky de Muckadell, 1980). The secretin cells in the proximal duodenum are thought to be stimulated by the acidic material from the stomach. The secretin cells in the distal duodenum and jejunum may respond to other, as yet undefined stimuli since, here, pH changes probably do not play a significant role.

Secretin was thought to occur in the S cell by virtue of its characteristic regional and topographic distribution (Bussolati et al., 1971; Polak et al., 1971b; Solcia et al., 1972). Direct ultrastructural identification of the secretin cells was made

possible by the consecutive semithin/ultrathin section technique (Paper II). The secretin cells of cat and pig duodenum were found to contain granules which are electron dense and pleiomorphic, often having a narrow electron lucent halo between the dense core and the limiting membrane (Fig.8). Sometimes the cells were seen to reach the lumen via an apical process. The luminal surface is covered with microvilli providing direct contact with the intestinal content (Fig. 2).

Neurotensin

Neurotensin is a tridecapeptide first isolated from the hypothalamus (Carraway and Leeman, 1973). It has been sequenced (Carraway and Leeman, 1975a) and synthesized (Carraway and Leeman, 1975b). Neurotensin affects intestinal and vascular smooth muscle tone, glycogenolysis, glucagon and insulin release and gastric acid secretion (Brown and Vale, 1976; Carraway et al., 1976a; Andersson et al., 1976; Rökaeus et al., 1977; Nagai and Frohman, 1978; Lundquist et al., 1979). Although neurotensin was originally isolated from the hypothalamus, it was soon found that more than 80% of neurotensin in the body is localized to the intestine (Carraway and Leeman, 1976b). Thus, neurotensin is one of the many peptides having a dual localization to brain and gut.

Orci et al. (1976) described neurotensin immunoreactive cells in canine ileum. The predominance of neurotensin cells in jejunum-ileum was confirmed in a study comprising several mammals (Paper III). In the cat, dog, and man the cells are fairly numerous; they are less frequent in the rat and guinea-pig and scarce in rabbit and pig. The cells are most numerous in the ileum, less so in the jejunum, and scarce in the duodenum and in the proximal colon. They were found to be absent from the stomach and pancreas. In most species the cells are situated on the villi, but in the dog and cat they are randomly distributed in the mucosa. The neurotensin cells are of the open type (Fig. 2) (Paper III; Frigerio et al., 1977; Helmstaedter et al., 1977b; Polak et al., 1977 ; Reinecke et al., 1980).

In the chicken, neurotensin cells were found all along the intestines (Reinecke et al., 1980; Paper III) but they are most numerous in the gizzard-duodenal junction (antrum). Neurotensin immunoreactive cells are few but regularly found in the colon and caeca, whereas they are absent from the proventriculus and pancreas.

The neurotensin cell was identified ultrastructurally using the consecutive semithin/ultrathin section technique (Paper IV). The mammalian neurotensin cell was

found to contain highly electron dense granules with a tightly applied membrane (Fig.8). Thus, the cells were indistinguishable from L cells, previously assumed to be a single cell type storing gut type glucagon (Paper IV; Polak et al., 1977). PAP staining of ultrathin sections revealed neurotensin to be contained within the cytoplasmic granules.

In the chicken antrum the neurotensin cells contain highly electron dense granules with a zone of rather low electron density between the dense core and the limiting membrane. The neurotensin cells are distinct from the gastrin and somatostatin cells, which also are abundant in this location.

Somatostatin

Somatostatin is a cyclic tetradecapeptide. It was isolated from the hypothalamus by virtue of its ability to inhibit the secretion of growth hormone (Brazeau et al., 1973). Immunocytochemical studies showed somatostatin to occur not only in the brain (Dubois et al., 1974; Hökfelt et al., 1974, 1975a; Pelletier et al., 1974) but also in endocrine cells and nerve fibers in the gut and pancreas (Luft et al., 1974; Dubois, 1975; Hökfelt et al., 1975a, b; Orci et al., 1975; Pelletier, 1975; Polak et al., 1975; Rufener et al., 1975b). A third type besides the insulin and glucagon cells was early recognized in the pancreatic islets (Bloom, 1931; cf. Fujita, 1968) and referred to as the A₁-cell (Hellerström and Hellman, 1960; cf. Grimelius and Wilander, 1980). On the basis of immunocytochemical and ultrastructural studies it was shown that this third cell type stored somatostatin (Goldsmith et al., 1975; Orci et al., 1975; Rufener et al., 1975a; Leclerc et al., 1976; cf. Orci et al., 1976a; Paper V). In the pancreatic islets of most mammalian and avian species the somatostatin cells have a peripheral location occurring disseminated among the glucagon cells. In addition some cells are found scattered in the central core of the islets.

Somatostatin cells are numerous in the gastric mucosa of all mammals (Paper V). In some species, notably cat, dog and man they predominate in the antrum, while in rabbit and pig they are most numerous in the oxyntic gland area. In the rat the somatostatin cells are fairly uniformly distributed in the whole of the stomach. In the duodenum, somatostatin cells are fairly numerous in cat, pig and man, but few in rat, rabbit and dog. In the remainder of the intestines, somatostatin cells are scarce. The somatostatin cells in the oxyntic gland area are of the closed type whereas in the

antrum and intestines they are of the open type (Fig. 2). In the chicken (cf. Orci et al., 1975 ; Paper V) somatostatin cells are numerous in the gizzard-duodenal junction (antrum) and in the proventriculus. They are scarce in the duodenum and absent from the rest of the gut.

In the pancreas and stomach the somatostatin cells have a characteristic morphology (Fig. 3). They possess long and slender processes ending in club-like swellings, often in close contact with neighbouring cells (Alumets et al., 1979a; Kusumoto et al., 1979; Larsson et al., 1979). The somatostatin cells are thought to influence neighbouring cells in a paracrine manner. The somatostatin granules differ in size from one species to another (Fig.8). The granules are rounded and have a fine-granulated dense core of moderate electron density. The limiting membrane is tightly applied to the dense core.

Freeze-dried paraffin embedded material is not suitable for the demonstration of somatostatin in neuronal elements in the gut (Paper V). Following formaldehyde perfusion and cryostat sectioning somatostatin containing nerve fibers can be readily demonstrated (Hökfelt et al., 1975 a,b; Sundler et al., 1980; Leander et al., 1981). In most species somatostatin immunoreactive nerves are rather few in the gut. They occur in the lamina propria around the basal parts of the glands, in the smooth muscle, and in the intramural plexuses.

Enkephalin

The discovery of specific opiate receptors in the CNS suggested the existence of endogenous ligands (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973; for a review see Frederickson, 1977). Hughes et al. (1975) identified a pair of pentapeptides in the brain and gut with potent opiate agonist activity. The two peptides, methionine (Met)- and leucine (Leu)-enkephalin, differ only in their COOH-terminal amino acid residue. The enkephalins are now known to belong to a large family of opioid peptides. Thus, the pituitary peptide β -lipotropin (β -LPH) contains the Met-enkephalin sequence (Lazarus et al., 1976). β -LPH does not bind to opiate receptors, but several Met-enkephalin-containing fragments of β -LPH do. They are referred to as endorphins (Ling et al., 1976).

Immunocytochemical studies revealed enkephalin immunoreactive nerve fibers not only in the brain but also in the gut wall of several species (Elde et al., 1976;

Polak et al., 1977 ; Linnoila et al., 1978; Schultzberg et al., 1978a; Paper VI). Such fibers are found all along the gut in all species studied. Most fibers are located in the myenteric plexus and in the smooth muscle layers. Immunoreactive nerve cell bodies are occasionally observed in the myenteric plexus. The enkephalin fibers seem to be intrinsic to the gut wall since they persist after autotransplantation (Malmfors et al., 1980, 1981). Enkephalin fibers are not restricted to the gut but occurs also in various autonomic ganglia (Schultzberg et al., 1978b, 1979 ; Alumets et al., 1979b; Pelto-Huikko, 1980). In the adrenal gland, immunoreactive enkephalin occurs both in nerve fibers and in medullary cells (Schultzberg et al., 1978b,c; Alumets et al., 1979b).

Immunoreactive enkephalin occurs in endocrine cells of the antrum and/or duodenum in certain species (Paper VI). The cells, constituting a sub-population of enterochromaffin cells, are particularly numerous in the pig. In the chicken, mouse, rat, rabbit, and monkey, enkephalin immunoreactive cells are few.

Ultrastructurally, the enkephalin immunoreactive cells in the porcine duodenum contain granules typical of enterochromaffin cells (Fig.8) (Paper VI). The immunoreactive material is stored in the granules exclusively, as shown by PAP staining of ultrathin sections.

Substance P

Substance P was originally extracted from brain and intestine by von Euler and Gaddum (1931) by virtue of its hypotensive and smooth muscle contractile effects. It was isolated from porcine hypothalamus and shown to be an undecapeptide (Chang and Leeman, 1970).

Substance P-containing nerve fibers have a wide-spread distribution in the body (cf. Hökfelt et al., 1980; cf. Brodin, 1981). Immunocytochemical studies have revealed a dual localization of substance P to nerve fibers and to endocrine cells in the gut (Nilsson et al., 1975; Pearse and Polak, 1975; Håkanson et al., 1977; Sundler et al., 1977b). Most of the substance P-containing nerve fibers occur in the myenteric plexus and the smooth muscle layers. In most species substance P containing nerve cell bodies are found only after colchicine treatment. Most, if not all, of the substance P immunoreactive nerve fibers in the gut are intrinsic to the gut (Franco et al., 1979; Malmfors et al., 1980, 1981; Schultzberg et al., 1978a).

Substance P immunoreactive endocrine cells do not seem to occur in the gut of all species. In the mouse and rat the cells are widely distributed in both small and large intestines (Sundler et al., 1977b), whereas in man they are restricted to the lower small intestine (unpublished observations). In still other species, such as the cat and pig, substance P cells seem to be absent from the gut. By applying techniques for amine fluorescence and immunocytochemistry sequentially on one and the same section, it was found that the substance P-containing cells in the mouse colon are identical with a sub-population of the 5-HT-containing enterochromaffin cells (Paper VII). Similar results have been obtained by others (Heitz et al., 1976, 1977).

Recent observations suggest the existence of immunoreactive physalaemin in the gut (Lazarus et al., 1980). Since most substance P antisera are directed against the COOH-terminus, which is common to substance P and other tachykinins (including physalaemin), they may crossreact with several members of this group of peptides (Fig.4). Region specific antisera are therefore necessary for the exact localization of the various tachykinins.

Ontogeny

The ontogeny of endocrine cells in the gastro-entero-pancreatic (GEP) region has been studied by several investigators (Björkman et al., 1966; Like and Orci, 1972; Larsson et al., 1974; Dubois et al., 1975; Larsson et al., 1975b, 1976; Dubois et al., 1976; Schweisthal et al., 1976; Helmstaedter et al., 1977a; Larsson, 1977a, b; Sundler et al., 1977a; Larsson and Jørgensen, 1978; Paulin and Dubois, 1978; Fuji, 1979; Lehy and Cristina, 1979; Track et al., 1979; Iwanaga et al., 1980; Papers II, III, V, VIII). Most reports deal with the cells of the pancreatic islets, but the development of endocrine cell types of the gut has also received attention. Various conventional histological or histochemical techniques have been used but during the last 10 years they have been gradually replaced by immunocytochemical methods. The latter often detect endocrine cells earlier than do the other techniques (Schweistahl et al., 1976).

In the porcine fetus (Paper VIII) GEP endocrine cells appear first in the pancreas. Insulin, glucagon and somatostatin cells occur in the dorsal pancreatic primordium, whereas PP cells are confined to the ventral primordium. At the 4 week stage (the earliest stage studied) endocrine cells seem to make up the bulk of the two pancreatic primordia and no ducts or acini are seen. During subsequent stages the endocrine cells are dispersed as if separated by the growing exocrine parenchyma, and during the 8-10 week gestational period the endocrine cells occur scattered in the pancreatic parenchyma. Later on, they start to form small aggregates. These small nests or islets increase gradually in size until the adult distribution pattern is established a few weeks after birth. Now, the endocrine cells form mantle islets with glucagon and somatostatin cells and an occasional PP cell in the periphery and with insulin cells in the central core; this type of islet is typical of the splenic part of the pancreas. In the duodenal part the mantle is formed predominantly by PP cells and somatostatin cells, whereas the glucagon cells are scarce (Bencosme and Liepa, 1955; Baetens et al., 1979; Rahier et al., 1979).

The first endocrine cell type to appear in the porcine gastro-intestinal tract seems to be the glucagon cell. At the 4 week stage glucagon cells are found in the upper small intestine close to the pancreatic primordia; they are localized in the basal part of the stratified intestinal epithelium. Subsequently, the glucagon cells in the duodenum increase in number but before parturition they disappear from this location

altogether. A few weeks after their appearance in the upper small intestine glucagon cells are found also in the oxyntic gland area of the stomach and in the jejunum-ileum and colon. In these latter locations they remain throughout development. After the glucagon cells the somatostatin and gastrin cells are next to appear. They are first seen in the distal stomach but within a few weeks they are also found in the small intestine. Somatostatin cells appear also in the large intestine, where they are first seen at the 7 week stage.

By using gastrin antisera with different region specificity it could be shown that in the porcine fetuses almost all cells in the antrum contained gastrin-17 whereas many of the cells in the small intestine, demonstrated with the COOH-terminal directed antiserum, seem to store CCK-like peptides rather than gastrin (cf. Larsson et al., 1977b). In the rat gastrin immunoreactive cells appear earlier in the duodenum than in the antrum (Larsson et al., 1974, 1976). In the pig, however, such cells are first found in the distal stomach. Furthermore, in the rat gastrin immunoreactive cells are found during a short period at birth in the pancreas (Larsson et al., 1974, 1976; Fujii, 1979). No such cells were detected in the pancreas of the pig.

Several endocrine cell types seem to appear first in the most proximal part of the small intestine, later to invade other regions of the gut (see also Larsson et al., 1974; Chayvialle et al., 1980). In the porcine fetus this is true not only for CCK and glucagon cells but also for those storing secretin, motilin or GIP. These three latter cell types, and the neurotensin cells, are restricted to the small intestine throughout development. Secretin, motilin and GIP cells appear first in the duodenum (at the 6-8 week stage) whereas neurotensin cells appear first in the distal small intestine (at the 8 week stage). Enkephalin cells appear rather late during porcine life compared to other endocrine cell types. They are first observed in the duodenum a few weeks before birth - and in the antrum a few days after birth. In the pancreas enkephalin cells are found immediately before birth, and they seem to predominate in the duodenal lobe. Here, the cells occur scattered in the exocrine parenchyma.

The functional significance of the early appearance of the various peptide hormones in the gut is unknown. Conceivably they participate in the initiation of secretion and motility of the gut (cf. Enochs and Johnson, 1977; Grasso et al., 1980). In addition, some peptides, notably gastrin and CCK, seem to have trophic effects on the gastro-intestinal tract (Johnson, 1979; Oscarson et al., 1979), while others, such as secretin,

have been claimed to be antitrophic (Johnson and Grossman, 1969). Possibly, the various gut hormones are functionally integrated also during ontogeny and act in concert to regulate the development and differentiation of the gut and its derivatives.

In view of the possibility that ontogeny is a rerun of phylogeny (Haeckel's bio-genetic rule) there is reason to compare the order of appearance of the various GEP hormones with available information on their phylogeny. With regard to the islet hormones insulin, glucagon, somatostatin and PP, which were present in the pancreatic buds already at the earliest stage studied, they are considered to be phylogenetically old (cf. Falkmer et al., 1981). The first islet organ occurs in the cyclostomes, i.e. the hagfish and the lamprey. In the Atlantic hagfish the islet organ is devoid of exocrine parenchyma and contains insulin cells (99%) and somatostatin cells (1%). The first compact exocrine pancreas equipped with islets of Langerhans occurs in the holocephalan ratfish. The endocrine pancreas is composed of insulin cells, somatostatin cells and glucagon cells. The ratfish PP cells remain in the gut mucosa. In the elasmobranchian cartilaginous fish the endocrine pancreas contains also numerous PP cells, constituting the first islet organ in evolution containing all four islet hormones. As all four hormones exist in the porcine pancreatic primordia already at the earliest stage studied (Paper VIII), their order of appearance cannot be deduced from the present findings. From the phylogenetic data, however, it can be speculated that they appear in the porcine fetus in the following sequence: insulin cells, somatostatin cells, glucagon cells and PP cells. Further studies are required to confirm this assumption. Hormones of the duodenum such as secretin, GIP and motilin on the other hand, appeared later than the islet hormones during gestation and these peptides seem to be phylogenetically young (Van Noorden and Falkmer, 1980; Falkmer et al., 1981).

TUMOURS STORING NEUROHORMONAL PEPTIDES IN THE GUT, PANCREAS AND OVARY

Peptide hormone-producing tumours have several histopathological features in common (Jouanneau and Malafosse, 1971; Soga and Tazawa, 1971; Dawson, 1976). The tumour cells form nests or strands separated by connective tissue. The cells are small and uniform in size and shape; mitoses are rare. Although the endocrine tumours deriving from foregut, midgut and hindgut share these features, they differ characteristically from each other with regard to their content of neurohormonal peptides, the spectrum of peptides reflecting their origin (Table IV-VI). Thus, foregut tumours may contain immunoreactive gastrin, insulin, glucagon, somatostatin, PP, VIP, calcitonin or enkephalin. Midgut tumours, on the other hand, often contain substance P, and hindgut tumours contain predominantly gut-type glucagon, somatostatin, PP or substance P.

Most endocrine tumours of foregut origin contain more than one neurohormonal peptide-producing cell type (Larsson et al., 1975; Sundler et al., 1979; Wilander et al., 1979a; Creutzfeldt, 1980b; Paper IX). Usually only one of the peptides gives rise to symptoms. Most pancreatic endocrine tumours contain demonstrable neurohormonal peptides. In several of the tumours the cells storing demonstrable peptides make up the majority cell population. Among these peptides are insulin, glucagon, gastrin, PP, somatostatin and VIP. Pancreatic endocrine tumours producing insulin, glucagon (cf. Holst, 1979), gastrin (cf. Zollinger et al., 1980) or VIP (cf. Morrison, 1980) usually give rise to specific constellations of symptoms. Gastrin-producing tumours in the stomach, however, do not always cause symptoms of hypergastrinemia. Endocrine tumours of the duodenum and upper jejunum often contain gastrin cells and somatostatin cells in varying proportions (DeLellis et al., 1976; Alumets et al., 1978; Kaneko et al., 1979); the gastrin producing tumours sometimes give rise to the Zollinger-Ellison syndrome.

Tumours arising from midgut derivatives are thought to be derived from enterochromaffin cells. Thus, they are rich in 5-hydroxytryptamine (5-HT), and those arising in the ileum are often associated with the "carcinoid syndrome" characterized by flushing of the skin, diarrhea, broncho-constriction and sudden drops in blood pressure (cf. Grahame-Smith, 1972; Martin and Potet, 1974; Dawson, 1976). Immunoreactive substance P is commonly found in midgut carcinoids (Håkanson et al., 1977; Wilander

Table IV

Immunoreactive peptides in endocrine cells in normal human tissue	Foregut derivatives	Immunoreactive peptides in tumours
calcitonin	<u>Thyroid</u>	calcitonin somatostatin ACTH
bombesin	<u>Respiratory tract</u>	ACTH calcitonin β -endorphin enkephalin GH HCG α -MSH neurophysin
gastrin glucagon somatostatin (enkephalin)	<u>Stomach</u>	gastrin
glucagon insulin pancreatic polypeptide somatostatin	<u>Pancreas</u>	glucagon insulin pancreatic polypeptide somatostatin gastrin VIP calcitonin neurotensin
gastrin somatostatin CCK secretin motilin GIP neurotensin substance P	<u>Duodenum and upper jejunum</u>	gastrin somatostatin

et al., 1979b). By isolating the granules from an intestinal argentaffin carcinoid we could show that these granules contain both substance P and 5-HT (Paper X). The granules are argentaffin reflecting the presence of 5-HT. Ultrastructurally, they are electron-dense and pleiomorphic (Fig. 8).

Table V

Immunoreactive peptides
in endocrine cells in
normal human tissue

Midgut derivatives

Immunoreactive
peptides in
tumours

substance P
glucagon (gut-type)
CCK
GIP
motilin
neurotensin
secretin
somatostatin
gastrin?

Lower jejunum,
ileum and
proximal colon

substance P
glucagon (gut-type)

Hindgut endocrine tumours are often multihormonal but rarely give rise to spectacular endocrine symptoms (Wilander et al., 1977a; Alumets et al., 1980; Fiocca et al., 1980; Paper XI). They seem to have a low tendency to give metastases and have generally a rather benign clinical course. The majority of the hindgut tumours display a trabecular or ribbon-like growth pattern and they are usually argyrophil and non-argentaffin. Most rectal endocrine tumours contain neurohormonal peptides, such as PP, gut-type glucagon, somatostatin, substance P, enkephalin, β -endorphin, and insulin immunoreactive material in decreasing order of frequency (Table VI) (Paper XI).

Table VI

Immunoreactive peptides
in endocrine cells in
normal human tissue

Hindgut derivatives

Immunoreactive
peptides in
tumours

glucagon (gut-type)
somatostatin
pancreatic polypeptide

Distal colon
and rectum

glucagon (gut-type)
somatostatin
pancreatic polypeptide
substance P
enkephalin
 β -endorphin
insulin

Tumours made up of peptide hormone-containing cells may arise in the ovary (ovarian carcinoids). Eighty-one primary ovarian carcinoids were analysed by immuno-histochemistry (Paper XII). From their spectrum of neurohormonal peptides they seem to be related to hindgut endocrine tumours. Gut endocrine tumours are thought to arise from peptide hormone-producing cells, which are normal constituents of the intestinal epithelium. However, in the female genital tract, such cells occur only in the vaginal, and, on rare occasions, in the endocervical epithelium. Primary ovarian carcinoids probably arise from peptide hormone-producing cells which occur in teratomatous tissue. Alternatively, cells programmed to produce neurohormonal peptides arise in the neural crest and migrate to the ovary where they later give rise to a carcinoid tumour.

Also tumours having an exocrine derivation may contain scattered peptide hormone-containing endocrine cells. In a study of such endocrine cells in mucinous cystadenocarcinomas of the ovary it was shown that their content of neurohormonal peptides resembles the spectrum of peptides encountered in the foregut (Paper XIII). A cytogenetic relationship between endocrine cells and mucin producing cells has been suggested from observations on mixed endocrine/exocrine tumours (adenocarcinoids) of the gut, notably appendix (Olsson and Ljungberg, 1980; Ratzenhofer and Auböck, 1980), on mucoid carcinoma of the breast (Fisher and Palekar, 1979) and on colonic adenocarcinomas of animals (Swartzendruber and Richter, 1980).

The classification of endocrine tumours may be based on either clinical or histopathological data. Endocrine tumours of foregut and midgut origin often give rise to spectacular symptoms (Paper IX), whereas hindgut endocrine tumours as a rule are clinically silent (Paper XI). To classify some endocrine tumours after the symptoms they give and others after their neurohormonal content seems illogical. This would favour a classification on histopathological grounds. Multihormonal tumours, however, represent a problem in the histopathological classification. To complicate matters further there are cases of multihormonal tumours in which a minority cell population causes the symptoms (Alumets et al., 1978). A reasonable solution of the problem is to characterize a GEP endocrine tumour by means of the following histopathological and clinical features:

1. Its histopathology (degree of differentiation, invasive growth, metastases), including information on the presence of argentaffin and/or argyrophil cells;

2. Its site of origin;
3. Its spectrum of GEP neurohormonal peptides;
4. Its association with signs or symptoms of endocrine disorder .

SUMMARY

The distribution, ultrastructure and ontogeny of various endocrine cell types in the gastro-entero-pancreatic (GEP) region was studied in several species by means of immunocytochemistry in combination with electron microscopy. In addition, the spectrum of neurohormonal peptides in endocrine tumours of foregut and hindgut derivatives, and in ovarian carcinoids and mucinous cystadenocarcinomas was studied.

1. Pancreatic-type glucagon was localized to a cell type of the dog stomach ultrastructurally indistinguishable from the pancreatic A cells, and contained round, membrane-bound cytoplasmatic granules of high electron density. Secretin cells were localized in the upper small intestine. These cells were ultrastructurally defined by their content of electron dense, slightly pleiomorphic granules. Mammalian neurotensin cells predominated in the distal small intestine and contained fairly large highly electron dense granules. Somatostatin cells were found throughout the gut and in the pancreas; the secretory granules were characterized by a fine-grained core of moderate electron density. Enkephalin cells were numerous in the antrum and duodenum of the pig, but scarce or absent in other species. In the pig the enkephalin cells contained 5-hydroxytryptamine (5-HT). The granules were rounded or pleiomorphic and highly electron dense. Also substance P was localized to a population of enterochromaffin cells as studied in the mouse gut.
2. A comprehensive study of the ontogeny of various GEP endocrine cells was performed in the pig. Insulin, glucagon, and somatostatin cells first appeared in the dorsal pancreatic primordium, whereas pancreatic polypeptide-containing cells first occurred in the ventral pancreatic primordium. Glucagon cells were the first endocrine cells to appear in the gastro-intestinal tract (duodenum). Gastrin cells and somatostatin cells appeared earlier in antrum than in the duodenum. Secretin, motilin, GIP and neurotensin cells were restricted to the small intestine during development; enkephalin cells appeared much later.
3. Endocrine tumours arising in foregut derivatives contain immunoreactive gastrin, insulin, glucagon, somatostatin, PP, VIP, calcitonin and neurotensin. Many of these tumours give rise to specific endocrine symptoms. Midgut endocrine tumours are derived from enterochromaffin cells and often contain substance P. Many of them are associated with the carcinoid syndrome. Hindgut endocrine tumours rarely cause endocrine symptoms; nevertheless they may contain several neurohormonal peptides such as PP, gut-

type glucagon, somatostatin, substance P, enkephalin, β -endorphin and/or insulin.

4. Ovarian carcinoids harbour neurohormonal peptides usually found in hindgut endocrine tumours. Such peptides are glucagon, enkephalin, somatostatin and substance P. Many argyrophil cells in cystadenocarcinomas display neurohormonal peptides: somatostatin, glucagon, gastrin/CCK, neurotensin and enkephalin.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to:

Maria Andersson, Eva Engelbert, Kristine Fogelström, Marianne Maxe-Colschen and Britt-Marie Svensson at the Department of Histology in Lund, Marianne Carlessen and Britt Carlsson at the Department of Pharmacology in Lund, and Leni Grubb, Birgitta Kjellander and Elise Nilsson at the Department of Pathology in Malmö for skilful technical assistance;

Hjalmar Gustavsson, Ragnar Mårtensson, Inger Norling, Agneta Persson and Bengt Wängelin for production of excellent illustrations;

Pia Bengtsson, Siv Carlson and Ingrid Stedman at the Department of Histology in Lund and Inger Ahlström and Kerstin Levander at the Department of Pathology in Malmö for expert secretarial work.

This work was supported by grants from the Medical Faculty, University of Lund, the Swedish Medical Research Council (Project Nos. 04X-1007, 04X-4499, 12X-718), Albert Pahlsson's Foundation, the Swedish Cancer Society (Project No. 806-05XA) and the Cancer Research Foundation at Malmö General Hospital.

REFERENCES

- Alumets, J., Ekelund, M., El Munshid, H.A., Håkanson, R., Lorén, I. and Sundler, F.: Topography of somatostatin cells in the stomach of the rat: possible functional significance. *Cell Tissue Res.* 202, 177-188, 1979a
- Alumets, J., Ekelund, G., Håkanson, R., Ljungberg, O., Ljungqvist, U., Sundler, F. and Tibblin, S.: Jejunal endocrine tumour composed of somatostatin and gastrin cells and associated with duodenal ulcer disease. *Virchows Arch. A. Path. Anat. and Histol.* 378, 17-22, 1978
- Alumets, J., Falkmer, S., Grimelius, L., Håkanson, R., Ljungberg, O., Sundler, F. and Wilander, E.: Immunocytochemical demonstration of enkephalin and β -endorphin in endocrine tumors of the rectum. *Acta path. microbiol. scand. Sect. A.* 88, 103-109, 1980
- Alumets, J., Håkanson, R., Malmfors, G. and Sundler, F.: Enkephalin in peripheral nerves, endocrine cells and endocrine tumours. An immunohistochemical study. *Molecular Endocrinology*. Eds. MacIntyre and Szelke, Elsevier/North-Holland Biomedical Press, 1979b, pp. 77-90
- Andersson, S., Chang, D., Folkers, K. and Rosell, S.: Inhibition of gastric acid secretion in dogs by neurotensin. *Life Sci.* 19, 367-370, 1976
- Baetens, D., Malaisse-Lagae, F., Perrelet, A. and Orci, L.: Endocrine pancreas: Three-Dimensional reconstruction shows two types of islets of Langerhans. *Science* 206, 1323-1325, 1979
- Baetens, D., Ruffener, C., Srikant, B.C., Dobbs, R., Unger, R. and Orci, L.: Identification of glucagon-producing cells (A Cells) in dog gastric mucosa. *J. Cell. Biol.* 69, 455-464, 1976
- Baum, J., Simons, B.E., Jr., Unger, R. and Madison, L.L.: Localization of glucagon in the alpha cells in the pancreatic islet by immunofluorescent technics. *Diabetes* 11, 371-374, 1962
- Bayliss, W.M. and Starling, E.H.: The mechanism of pancreatic secretion. *J. Physiol.* 28, 325-353, 1902
- Beauvillain, J.C., Tramu, G., Dubois, M.P.: Characterization by different techniques of adrenocorticotropin and gonadotropin-producing cells in lerot pituitary (*Eliomys quercinus*). *Cell Tissue Res.* 158, 301-317, 1975
- Bencosme, S.A. and Liepa, E.: Regional differences of the pancreatic islet. *Endocrinology* 57, 588-593, 1955
- Björkman, N., Hellerström, B., Hellman, B. and Petersson, B.: The cell types in the human fetus. *Z. Zellforsch.* 72, 425-445, 1966
- Bloom, W.: A new type of granular cell in the islets of Langerhans of man. *Anat. Rec.* 49, 363-371, 1931
- Bodanszky, M., Ondetti, M.A., Levine, S.D. and Williams, N.J.: Synthesis of secretin. II. The stepwise approach. *J. Am. Chem. Soc.* 89, 6753-6757, 1967

- Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. and Guillemin, R.: Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* 179, 77-79, 1973
- Brodin, E.: Substance P, 1981 (Thesis)
- Bromer, W.W., Sinn, L.G., Staub, A., Behrens, O.K., Diller, E.R. and Bird, H.L.: The amino acid sequence of glucagon. V. Localization of amino groups, acid degradation studies, and summary of sequential evidence. *J. Am. Chem. Soc.* 79, 2807-2810, 1957
- Brown, J.C., Cook, M.A. and Dryburgh, J.R.: Motilin, a gastric motor activity stimulating polypeptide: the complete amino acid sequence. *Can. J. Biochem.* 51, 533-537, 1973
- Brown, J.C. and Dryburgh, J.R.: A gastric inhibitory polypeptide II: The complete amino acid sequence. *Can. J. Biochem.* 49, 867-872, 1971
- Brown, M. and Vale, W.: Effects of neurotensin and substance P on plasma insulin, glucagon and glucose levels. *Endocrinology* 98, 819-821, 1976
- Bussolati, G., Capella, C., Solcia, E., Vassallo, G. and Vezzadini, P.: Ultrastructural and immunofluorescent investigations on the secretin cell in the dog intestinal mucosa. *Histochemie* 26, 218-227, 1971
- Carraway, R.E., Demers, L.M. and Leeman, S.E.: Hyperglycemic effect of neurotensin, a hypothalamic peptide. *Endocrinology* 99, 1452-1462, 1976a
- Carraway, R. and Leeman, S.E.: The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalamus. *J. Biol. Chem.* 248, 6854-6861, 1973
- Carraway, R. and Leeman, S.E.: The amino acid sequence of a hypothalamic peptide, neurotensin. *J. Biol. Chem.* 250, 1907-1911, 1975a
- Carraway, R. and Leeman, S.E.: The synthesis of neurotensin. *J. Biol. Chem.* 250, 1912-1918, 1975b
- Carraway, R. and Leeman, S.E.: Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. *J. Biol. Chem.* 251, 7045-7052, 1976b
- Chance, R.E., Moon, N.E. and Johnson, M.G.: Human pancreatic polypeptide, (HPP) and bovine pancreatic polypeptide (BPP). In: *Methods of Hormone Radioimmunoassay*. Eds. B.M. Jaffe et al., Academic Press, New York, 1979, pp. 657-672.
- Chang, M.M. and Leeman, S.E.: Isolation of sialogogic peptide from bovine hypothalamic tissue and its characterization as substance P. *J. Biol. Chem.* 245, 4784-4790, 1970
- Chayvialle, J.A., Paulin, C., Dubois, P.M., Descos, F. and Dubois, M.P.: Ontogeny of somatostatin in the human gastro-intestinal tract, endocrine pancreas and hypothalamus. *Acta Endocr. (Kbh)* 94, 1-10, 1980
- Conlon, J.M.: The glucagon-like polypeptides - order out of chaos? *Diabetologia* 18, 85-88, 1980
- Conlon, J.M., Samson, W.K., Dobbs, R.E., Orci, L. and Unger, R.H.: Glucagon-like polypeptides in canine brain. *Diabetes* 28, 700-702, 1979

- Coons, A.H., Leduc, E.H. and Conolly, J.M.: Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* 102, 49-60, 1955
- Creutzfeldt, W. (Ed): Gastrointestinal hormones. *Clin. Gastroenterology* 9, 483-803, 1980a
- Creutzfeldt, W.: Endocrine tumors of the pancreas: clinical, chemical and morphological findings. *Int. Acad. Path., Monographs in pathology* 21, 208-230, 1980b
- Dawson, I.M.P.: The endocrine cells of the gastro-intestinal tract and the neoplasms which arise from them. *Current Topics in Pathology* 63, 222-258, 1976
- DeLellis, R.A., Gagel, R.F., Kaplan, M.M. and Curtis, L.E.: Gastrinoma of duodenal G-cell origin. *Cancer* 38, 201-208, 1976
- Dubois, M.P.: Immunoreactive somatostatin is present in discrete cells of the endocrine pancreas. *Proc. Nat. Anat. Sci USA* 72, 1340-1343, 1975
- Dubois, M.P., Barry, J. and Leonardelli, J.: Endocrinologie - Mise en évidence par immunofluorescence et répartition de la somatostatine (SRIF) dans l'émersion des Vertébrés (Mammifères, Oiseaux, Amphibiens, Poissons). *C.R. Acad. Sc. (Paris)* 279 D, 1899-1902, 1974
- Dubois, P.M., Paulin, C., Assan, R. and Dubois, M.P.: Evidence for immunoreactive somatostatin in the endocrine cells of human foetal pancreas. *Nature* 256, 731-732, 1975
- Dubois, P.M., Paulin, C. and Chayvialle, J.A.: Identification of gastrin-secreting cells and cholecystokinin-secreting cells in the gastrointestinal tract of the human fetus and adult man. *Cell Tissue Res.* 175, 351-356, 1976
- Edkins, J.S. and Cantab, M.B.: On the chemical mechanism of gastric secretion. *Proc. Royal Soc. (London)* 76, 376, 1905
- Elde, R., Hökfelt, T., Johansson, O. and Terenius, L.: Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. *Neuroscience* 1, 349-351, 1976
- Enochs, M.R. and Johnson, L.R.: Hormonal regulation of gastrointestinal tract growth; biochemical and physiologic aspects. *Progr. Gastroenter.* 3, 3-28, 1977
- Euler, U.S.v. and Gaddum, J.H.: An unidentified depressor substance in certain tissue extracts. *J. Physiol.* 72, 74-87, 1931
- Falkmer, S., Carraway, R.E., El-Salhy, M., Emdin, S.O., Grimelius, L., Rehfeld, J.F., Reinecke, M. and Schwartz, T.F.W.: Phylogeny of the gastroenteropancreatic neuroendocrine system. A review with special reference to the occurrence of CCK-like and neurotensin-like polypeptides in lower vertebrates and invertebrates. *UCLA Forum Med. Sci.* 23, 21-42, 1981
- Fiocca, R., Capella, C., Buffa, R., Fontana, R., Solcia, E., Hage, E., Chance, R.E. and Moody, A.J.: Glucagon-like, glicentin-like, and pancreatic polypeptide-like immunoreactivities in rectal carcinoids and related colorectal cells. *Am.J. Path.* 100, 81- 92, 1980

- Fisher, E.R. and Palekar, A.S.: Solid and mucinous varieties of so-called mammary carcinoid tumors. *Am.J.Clin.Pathol.* 72, 909-916, 1979
- Forssmann, W.G., Orci, L., Pictet, R., Renold, A.E. and Rouiller, C.: The endocrine cells in the epithelium of the gastrointestinal mucosa of the rat. An electron microscope study. *J. Cell Biol.* 40, 692-715, 1969
- Franco, R., Costa, M. and Furness, J.B.: Evidence that axons containing substance P in the guinea-pig ileum are of intrinsic origin. *Naunyn-Schmiedeberg's Arch Pharmacol.* 307, 57-63, 1979
- Frederickson, C.A.: Enkephalin pentapeptides - a review of current evidence for a physiological role in vertebrate neurotransmission. *Life Sci.* 21, 23-42, 1977
- Frigerio, B., Ravazola, M., Ito, S., Buffa, R., Capella, C., Solcia, E. and Orci, L.: Histochemical and ultrastructural identification of neurotensin cells in the dog ileum. *Histochemistry* 54, 123-131, 1977
- Fujii, S.: Development of pancreatic endocrine cells in the rat fetus. *Arch. histol. jap.* 42, 467-479, 1979
- Fujita, T.: D Cell, the third endocrine element of the pancreatic islet. *Arch. histol. jap.* 29, 1-40, 1968
- Goldsmith, P.C., Rose, J.C., Arimura, A. and Ganong, W.F.: Ultrastructural localization of somatostatin in pancreatic islets of the rat. *Endocrinology* 97, 1061-1064, 1975
- Grahame-Smith, D.G.: *The Carcinoid Syndrome*. William Heinemann Medical Books Ltd., London, 1972, pp. 1-96
- Grasso, S., Fallucca, F., Mazzone, D., Gerlini, G., Russo, A. and Reitano, G.: Insulin and glucagon secretion in the human fetus and newborn. *Proc. of the Sero Symposia* 30, 83-91, 1980
- Gregory, R.A. and Tracy, H.J.: The constitution and properties of two gastrins extracted from hog antral mucosa. Part I. The isolation of two gastrins from hog antral mucosa. *Gut* 5, 103-117, 1964
- Grimelius, L., Capella, C., Buffa, R., Polak, J.M., Pearse, A.G.E. and Solcia, E.: Cytochemical and ultrastructural differentiation of enteroglucagon and pancreatic-type glucagon cells of the gastrointestinal tract. *Virchows Arch. B Cell Path.* 20, 217-228, 1976
- Grimelius, L. and Wilander, E.: Silver stains in the study of endocrine cells of the gut and pancreas. *Invest. Cell Pathol.* 3, 3-12, 1980
- Håkanson, R., Bengmark, S., Brodin, E., Ingemansson, S., Larsson, L.-I., Nilsson, G. and Sundler, F.: Substance P-like immunoreactivity in intestinal carcinoid tumors. In: *Substance P*. Eds. U.S. von Euler and B. Pernow, Raven Press, New York, 1977, pp. 55-58.
- Harper, A.A. and Raper, H.S.: Pancreozymin, a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine. *J. Physiol.* 102, 115-125, 1943

- Heitz, Ph., Polak, J.M., Kasper, M., Timson, C.M. and Pearse, A.G.E.: Immunoelectroncytochemical localization of motilin and substance P in rabbit bile duct enterochromaffin cells. *Histochemistry* 50, 319-325, 1977
- Heitz, Ph., Polak, J.M., Timson, C.M. and Pearse, A.G.E.: Enterochromaffin cells as the endocrine source of gastrointestinal substance P. *Histochemistry* 49, 343-347, 1976
- Hellerström, C. and Hellman, B.: Some aspects of silver impregnation of the islet of Langerhans in the rat. *Acta endocr. (Kbh)* 35, 518-532, 1960
- Helmstaedter, V., Mühlmann, G., Feurle, G.E. and Forssmann, W.G.: Immunohistochemical identification of gastrointestinal neurotensin cells in human embryos. *Cell Tissue Res.* 184, 315-320, 1977a
- Helmstaedter, V., Taugner, Ch., Feurle, G.E. and Forssmann, W.G.: Localization of neurotensin-immunoreactive cells in the small intestine of man and various mammals. *Histochemistry* 53, 35-41, 1977b
- Hökfelt, T., Efendić, S., Hellerström, C., Johansson, O., Luft, R. and Arimura, A.: Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special references to the A₁-cells of the pancreatic islets and to the hypothalamus. *Acta endocr. (Copenh.) Suppl.* 200, 5-41, 1975a
- Hökfelt, T., Efendić, S., Johansson, O., Luft, R. and Arimura, A.: Immunohistochemical localization of somatostatin (growth hormone release-inhibiting factor) in the guinea pig brain. *Brain Res.* 80, 165-169, 1974
- Hökfelt, T., Johansson, O., Efendić, S., Luft, R. and Arimura, A.: Are there somatostatin-containing nerves in the rat gut? Immunohistochemical evidence for a new type of peripheral nerves. *Experientia* 31, 852-854, 1975
- Hökfelt, T., Johansson, O., Ljungdahl, Å., Lundberg, J.M. and Schultzberg, M.: Peptidergic neurones. *Nature* 284, 515-521, 1980
- Holst, J.J.: Extraction, gel filtration pattern, and receptor binding of porcine gastrointestinal glucagon-like immunoreactivity. *Diabetologia* 13, 159-169, 1977
- Holst, J.J.: Gut endocrine tumour syndromes. *Clin. Endocrinol. Metab.* 8, 413-432, 1979
- Holst, J.J.: Evidence that glicentin contains the entire sequence of glucagon. *Biochem. J.* 187, 337-343, 1980
- Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R.: Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258, 577-579, 1975
- Ivy, A.C. and Oldberg, E.: A hormone mechanism for gall-bladder contraction and evacuation. *Am.J.Physiol.* 86, 599-613, 1928
- Iwanaga, T., Kobayashi, S., Fujita, T. and Yanaihara, N.: Immunocytochemistry and ultrastructure of Segi's cap. *Biomed. Res.* 1, 117-129, 1980

- Jacobsen, H., Demandt, A., Moody, A.J. and Sundby, F.: Sequence analysis of porcine gut GLL-1. *Biochim. Biophys. Acta* 493, 452-459, 1977
- Johnson, L.R.: Regulation of gastrointestinal mucosal growth. *World J. Surg.* 3, 477-487, 1979
- Johnson, L.R. and Grossman, M.I.: Characteristics of inhibition of gastric secretion by secretin. *Am. J. Physiol.* 217, 1401-1404, 1969
- Jorpes, J.E. and Mutt, V.: On the biological activity and amino acid composition of secretin. *Acta Chem. Scand.* 15, 1779-1791, 1961
- Jouanneau, P. and Malafosse, M.: Les tumeurs carcinoïdes du tube digestif. *Monogr. Assoc. Franc. Chir.* 73, 1-193, 1971
- Kaneko, H., Yanaihara, N., Ito, S., Kusumoto, Y., Fujita, T., Ishikawa, S., Sumida, T. and Sekiya, M.: Somatostatinoma of the duodenum. *Cancer* 44, 2273-2279, 1979
- Kimmel, J.R., Pollock, H.G. and Hazelwood, R.L.: Isolation and characterization of chicken insulin. *Endocrinology* 83, 1323-1330, 1968
- Kubes, L., Jirásek, K. and Lomský, R.: Endocrine cells of the dog gastrointestinal mucosa. *Cytologia* 39, 179-194, 1974
- Kusumoto, Y., Iwanaga, T., Ito, S. and Fujita, T.: Juxtaposition of somatostatin cell and parietal cell in the dog stomach. *Arch. histol. jap.* 42, 459-465, 1979
- Larsson, L.-I.: Ontogeny of peptide-producing nerves and endocrine cells of the gastro-duodeno-pancreatic region. *Histochemistry* 54, 133-142, 1977a
- Larsson, L.-I., Goltermann, N., de Magistris, L., Rehfeld, J.F. and Schwartz, T.W.: Somatostatin cell processes as pathways for paracrine secretion. *Science* 205, 1393-1395, 1979
- Larsson, L.-I., Grimelius, L., Håkanson, R., Rehfeld, J.F., Stadil, F., Holst, J., Angervall, L. and Sundler, F.: Mixed endocrine pancreatic tumors producing several peptide hormones. *Am. J. Pathol.* 79, 271-284, 1975a
- Larsson, L.-I., Håkanson, R., Rehfeld, J.F., Stadil, F. and Sundler, F.: Occurrence and neonatal development of gastrin immunoreactivity in the digestive tract of the rat. *Cell Tissue Res.* 149, 275-281, 1974
- Larsson, L.-I., Håkanson, R., Sjöberg, N.-O. and Sundler, F.: Fluorescence histochemistry of the gastrin cell in fetal and adult man. *Gastroenterology* 68, 1152-1159, 1975b
- Larsson, L.-I., Holst, J., Håkanson, R. and Sundler, F.: Distribution and properties of glucagon immunoreactivity in the digestive tract of various mammals: An immunohistochemical and immunochemical study. *Histochemistry* 44, 281-299, 1975c
- Larsson, L.-I. and Jørgensen, L.M.: Ultrastructural and cytochemical studies on the cytodifferentiation of duodenal endocrine cells. *Cell Tissue Res.* 194, 79-102, 1978

- Larsson, L.-I., Rehfeld, J.F. and Goltermann, N.: Gastrin in the human fetus. Distribution and molecular forms of gastrin in the antro-pyloric gland area. Duodenum and pancreas. *Scand. J. Gastroent.* 12, 869-872, 1977b
- Larsson, L.-I., Rehfeld, J.F., Sundler, F. and Håkanson, R.: Pancreatic gastrin in foetal and neonatal rats. *Nature* 262, 609-610, 1976
- Lazarus, L.H., Ling, N. and Guillemin, R.: β -Lipotropin as a prohormone for the morphinomimetic peptides endorphins and enkephalins. *Proc. Natl. Acad. Sci. USA* 73, 2156-2159, 1976
- Lazarus, L.H., Linnoila, R.I., Hernandez, O. and Di Augustine, R.P.: A neuropeptide in mammalian tissues with physalaemin-like immunoreactivity. *Nature* 287, 555-558, 1980
- Leander, S., Håkanson, R. and Sundler, F.: Nerves containing substance P, vasoactive intestinal polypeptide, enkephalin or somatostatin in the guinea-pig taenia coli. Distribution, ultrastructure and possible functions. *Cell Tissue Res.* 215, 21-39, 1981
- Leclerc, R., Pelletier, G., Puviani, R., Arimura, A. and Schally, A.V.: Immunohistochemical localization of somatostatin in endocrine cells of the rat stomach. *Molec. Cell. Endocrinol.* 4, 257-261, 1976
- Lehy, T. and Cristina, M.L.: Ontogeny and distribution of certain endocrine cells in the human fetal large intestine. *Cell Tissue Res.* 203, 415-426, 1979
- Like, A.A. and Orci, L.: Embryogenesis of the human pancreatic islets: A light and electron microscopic study. *Diabetes* 21 (Suppl. 2) 511-534, 1972
- Ling, N., Burgus, R. and Guillemin, R.: Isolation, primary structure, and synthesis of α -endorphin and γ -endorphin, two peptides of hypothalamic-hypophysial origin with morphinomimetic activity. *Proc. Natl. Acad. Sci. USA* 73, 3942-3946, 1976
- Linnoila, K.L., DiAugustine, R.P., Miller, R.J., Chang, K.J. and Cuatrecasas, P.: An immunohistochemical and radioimmunological study of the distribution of (Met⁵)- and (Leu⁵)-enkephalin in the gastrointestinal tract. *Neuroscience* 3, 1187-1196, 1978
- Lorén, I., Alumets, J., Håkanson, R., Sundler, F. and Thorell, J.: Gut-type glucagon immunoreactivity in nerves of the rat brain. *Histochemistry* 61, 335-341, 1979
- Luft, R., Efendic, S., Hökfelt, T., Johansson, O. and Arimura, A.: Immunohistochemical evidence for the localization of somatostatin-like immunoreactivity in a cell population of the pancreatic islets. *Med. Biol.* 52, 428-430, 1974
- Lundquist, I., Sundler, F., Åhrén, B., Alumets, J. and Håkanson, R.: Somatostatin, pancreatic polypeptide, substance P, and neurotensin: Cellular distribution and effects on stimulated insulin secretion in the mouse. *Endocrinology* 104, 832-838, 1979
- Malmfors, G., Håkanson, R., Okmian, L. and Sundler, F.: Peptidergic nerves persist after jejunal autotransplantation: an experimental study in the piglet. *J. Ped. Surg.* 15, 53-56, 1980
- Malmfors, G., Leander, S., Brodin, E., Håkanson, R., Holmin, T. and Sundler, F.: Peptide-containing neurons intrinsic to the gut wall. An experimental study in the pig. *Cell Tissue Res.* 214, 225-238, 1981

Martin, E. and Potet, F.: Pathology of endocrine tumours of the GI tract. Clin. Gastroenterology 3, 511-532, 1974

Mayor, H.D., Hampton, J.C. and Rosario, R.: A simple method for removing the resin from epoxyembedded tissue. J. biophys. Cytol. 9, 909-910, 1961

Morrison, A.B.: Islet cell tumors and the diarrheogenic syndrome. The Cancer. Int. Acad. of Path., Monographs in Pathology 21, 185-207, 1980

Murlin, J.R., Clough, H.D., Gibbs, C.B.F. and Stokes, A.M.: Aqueous extracts of pancreas. 1. Influence on the carbohydrate metabolism of depancreatized animals. J. Biol. Chem. 56, 253-296, 1923

Mutt, V. and Jorpes, E.: Hormonal polypeptides of the upper intestine. Biochem. J. 125, 57-58P, 1971

Mutt, V., Jorpes, J.E. and Magnusson, S.: Structure of porcine secretin. The amino acid sequence. Eur. J. Biochem. 15, 513-519, 1970

Mutt, V., Magnusson, S., Jorpes, J.E. and Dahl, E.: Structure of porcine secretin. I. Degradation with trypsin and thrombin. Sequence of the tryptic peptides. The C-terminal residue. Biochemistry 4, 2358-2362, 1965

Mutt, V. and Said, S.I.: Structure of the porcine vasoactive intestinal octacosapeptide. The amino acid sequence. Use of kallikrein in its determination. Eur. J. Biochem. 42, 581-583, 1974

Nagai, K. and Frohman, L.A.: Neurotensin hyperglycemia: evidence for histamine mediation and the assessment of a possible physiologic role. Diabetes 27, 577-582, 1978

Nilsson, G., Larsson, L.-I., Håkanson, R., Brodin, E., Pernow, B. and Sundler, F.: Localization of substance P-like immunoreactivity in mouse gut. Histochemistry 43, 97-99, 1975

Olsson, B. and Ljungberg, O.: Adenocarcinoid of the vermiform appendix. Virchows Arch. A Path. Anat. and Histol. 386, 201-210, 1980

Orci, L., Baetens, D., Dubois, M.P. and Rufener, C.: Evidence for the D-Cell of the pancreas secreting somatostatin. Horm. Metab. Res. 7, 400-402, 1975

Orci, L., Baetens, D., Ravazzola, M., Malaisse-Lagae, F., Amherdt, M. and Rufener, C.: Somatostatin in the pancreas and the gastrointestinal tract. In: Endocrine gut and pancreas. Ed. T. Fujita, Elsevier, Amsterdam, 1976a, pp. 73-88.

Orci, L., Baetens, O., Rufener, C., Brown, M., Vale, W. and Guillemin, R.: Evidence for immunoreactive neurotensin in dog intestinal mucosa. Life Sci. 19, 559-562, 1976b

Orci, L., Pictet, R., Forssmann, W.G., Renold, A.E. and Rouiller, C.: Structural evidence for glucagon producing cells in the intestinal mucosa of the rat. Diabetologia 4, 56-67, 1968

Oscarson, J., Håkanson, R., Liedberg, G., Lundqvist, G., Sundler, F. and Thorell, J.: Variated serum gastrin concentration: Trophic effects on the gastrointestinal tract of the rat. *Acta Phys. Scand. Suppl.* 475, 1-27, 1979

Paulin, C. and Dubois, P.M.: Immunohistochemical identification and localization of pancreatic polypeptide cells in the pancreas and gastrointestinal tract of the human fetus and adult man. *Cell Tissue Res.* 188, 251-257, 1978

Pearse, A.G.E. and Polak, J.M.: Immunocytochemical localization of substance P in mammalian intestine. *Histochemistry* 41, 373-375, 1975

Pelletier, G., Labrie, F., Arimura, A. and Schally, A.V.: Electron microscopic immunohistochemical localization of growth hormone-release inhibiting hormone (somatostatin) in the rat median eminence. *Am. J. Anat.* 140, 445-450, 1974

Pelletier, G., Leclerc, R., Arimura, A. and Schally, A.V.: Immunohistochemical localization of somatostatin in the rat pancreas. *J. Histochem. Cytochem.* 23, 699-701, 1975

Pelto-Huikko, M., Hervonen, A., Helen, P., Linnoila, I., Pickel, V.M. and Miller, R.J.: Localization of (Met⁵)- and (Leu⁵)-enkephalin in nerve terminals and SIF cells in adult human sympathetic ganglia. In: *Histochemistry and Cell Biology of Autonomic Neurons, SIF Cells, and Paraneurons*. Eds. O. Eränkö et al., Raven Press, New York, 1980, pp. 379-383,

Pert, C.B. and Snyder, S.H.: Opiate receptor: demonstration in nervous tissue. *Science* 179, 1011-1014, 1973

Polak, J.M., Bloom, S., Coulling, I. and Pearse, A.G.E.: Immunofluorescent localization of enteroglucagon cells in the gastrointestinal tract of the dog. *Gut* 12, 311-318, 1971a

Polak, J.M., Coulling, I., Bloom, S. and Pearse, A.G.E.: Immunofluorescent localization of secretin and enteroglucagon in human intestinal mucosa. *Scand. J. Gastroent.* 6, 739-744, 1971b

Polak, J.M., Pearse, A.G.E., Grimelius, L., Bloom, S.R. and Arimura, A.: Growth-hormone release-inhibiting hormone in gastrointestinal and pancreatic D cells. *Lancet*, 1220-1222, 1975

Polak, J.M., Sullivan, S.N., Bloom, S.R., Buchan, A.M.J., Facer, P., Brown, M.R. and Pearse, A.G.E.: Specific localisation of neurotensin to the N cell in human intestine by radioimmunoassay and immunocytochemistry. *Nature* 270, 183-184, 1977a

Polak, J.M., Sullivan, S.N., Bloom, S.R., Facer, P. and Pearse, A.G.E.: Enkephalin-like immunoreactivity in the human gastrointestinal tract. *Lancet* 972-974, 1977b

Rahier, J., Wallon, J., Gepts, W. and Haot, J.: Localization of pancreatic polypeptide cells in a limited lobe of the human neonate pancreas: remnant of the ventral primordium? *Cell Tissue Res.* 200, 359-366, 1979

Rahier, J., Wallon, J. and Henquin, J.C.: Abundance of somatostatin cells in the human neonatal pancreas. *Diabetologia* 18, 251-254, 1980

Ratzenhofer, M. and Auböck, L.: The amphicrine (endo-exocrine) cells in the human gut, with a short reference to amphicrine neoplasias. *Acta Morph. Acad. Sci. Hung.* 28, 37-58, 1980

Ravazzola, M. and Orci, L.: Glucagon and glicentin immunoreactivity are topologically segregated in the α granule of the human pancreatic A cell. *Nature* 284, 66-67, 1980

Ravazzola, M., Siperstein, A., Moody, A.J., Sundby, F., Jacobsen, H. and Orci, L.: Glicentin immunoreactive cells: their relationship to glucagon-producing cells. *Endocrinology* 105, 499-508, 1979

Rehfeld, J.F.: Heterogeneity of gastrointestinal hormones. *World J. Surg.* 3, 415-422, 1979

Reinecke, M., Almasan, K., Carraway, R., Helmstaedter, V. and Forssmann, W.G.: Distribution patterns of neurotensin-like immunoreactive cells in the gastro-intestinal tract of higher vertebrates. *Cell Tissue Res.* 205, 383-395, 1980

Rökæus, Å., Burcher, E., Chang, D., Folkers, K. and Rosell, S.: Actions of neurotensin and (Gln⁴)-neurotensin on isolated tissues. *Acta pharmacol. et toxicol.* 41, 141-147, 1977

Rufener, C., Amherdt, M., Dubois, M.P. and Orci, L.: Ultrastructural immunocytochemical localization of somatostatin in rat pancreatic monolayer culture. *J. Histochem. Cytochem.* 23, 866-869, 1975a

Rufener, C., Dubois, M.P., Malaisse-Lagae, F. and Orci, L.: Immuno-fluorescent reactivity to anti-somatostatin in the gastro-intestinal mucosa of the dog. *Diabetologia* 11, 321-324, 1975b

Sanger, F. and Thompson, E.O.P.: The amino-acid sequence in the glycyl chain of insulin. 1. The identification of lower peptides from partial hydrolysates. *Biochem. J.* 53, 353-374, 1953

Sasaki, H., Rubalcava, B., Baetens, D., Blazquez, E., Srikant, C.B., Orci, L. and Unger, H.: Identification of glucagon in the gastrointestinal tract. *J. Clin. Invest.* 56, 135-145, 1975

Schaffalitzky de Muckadell, O.B.: Secretin and pancreatic bicarbonate secretion in man. 1980 (Thesis)

Schaffalitzky de Muckadell, O.B. and Fahrenkrug, J.: Preparation of ¹²⁵I-labelled synthetic porcine secretin for radioimmunoassay. *Scand. J. clin. Lab. Invest.* 36, 661-668, 1976

Schultzberg, M., Dreyfus, C.F., Gershon, M.D., Hökfelt, T., Elde, R., Nilsson, G., Said, S. and Goldstein, M.: VIP-, enkephalin-, substance P-, and somatostatin-like immunoreactivity in neurons intrinsic to the intestine: Immunohistochemical evidence from organotypic tissue cultures. *Brain Res.* 155, 239-248, 1978a

Schultzberg, M., Hökfelt, T., Lundberg, J.M., Terenius, L., Elfvin, L.-G. and Elde, R.: Enkephalin-like immunoreactivity in nerve terminals in sympathetic ganglia and adrenal medulla and in adrenal medullary gland cells. *Acta physiol. scand.* 103, 475-477, 1978b

- Schultzberg, M., Hökfelt, T., Terenius, L., Elfvin, L.-G., Lundberg, J.M., Brandt, J., Elde, R.P. and Goldstein, M.: Enkephalin immunoreactive nerve fibres and cell bodies in sympathetic ganglia of the guinea-pig and rat. *Neuroscience* 4, 249-270, 1979
- Schultzberg, M., Lundberg, J.M., Hökfelt, T., Terenius, L., Brandt, J., Elde, R.P. and Goldstein, M.: Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. *Neuroscience* 3, 1169-1186, 1978c
- Schweisthal, M.R., Frost, C.C. and Brinn, J.E.: Ontogeny of four cell types in fetal rat islets using histochemical techniques. *Acta diabet. lat.* 13, 30-39, 1976
- Simon, E.J., Hiller, J.M. and Edelman, I.: Stereospecific binding of the potent narcotic analgesic (^3H) etorphine to rat-brain homogenate. *Proc. Nat. Acad. Sci. USA* 70, 1947-1949, 1973
- Soga, J.: Carcinoids: Their changing concepts and a new histologic classification. In: *Gastro-Entero-Pancreatic Endocrine System*. Ed. T. Fujita, Georg Thieme Publ., Stuttgart, 1974, pp. 101-119
- Soga, J. and Tazawa, K.: Pathologic analysis of carcinoids. *Cancer* 28, 990-998, 1971
- Solcia, E., Capella, C., Vassallo, G. and Buffa, R.: Endocrine cells of the gastric mucosa. *Int. Rev. Cytol.* 42, 223-286, 1975
- Solcia, E., Capella, C., Vezzadini, P., Barbara, L. and Bussolati, G.: Immunohistochemical and ultrastructural detection of the secretin cells in the pig intestinal mucosa. *Experientia* 28, 549-550, 1972
- Steiner, D.F.: Insulin precursors. In: *Structural studies on molecules of biological interest*. A volume in honour of Dorothy Hodgkin. Eds. G. Dodson et al., Clarendon Press, Oxford, 1981, pp. 407-419.
- Sternberger, L.A.: *Immunocytochemistry*. Prentice-Hall Inc., New Jersey, 1974
- Sundby, F., Jacobsen, H. and Moody, A.J.: Purification and characterization of a protein from porcine gut with glucagon-like immunoreactivity. *Horm. Metab. Res.* 8, 366-371, 1976
- Sundler, F., Alumets, J. and Håkanson, R.: Peptides in the gut with dual distribution in nerves and endocrine cells. In: *Gut Hormones*, Ed. S.R. Bloom. Churchill-Livingstone, 1978, pp. 406-413
- Sundler, F., Alumets, J. and Håkanson, R.: Majority and minority cell populations in GEP and bronchial endocrine tumours. *Scand. J. Gastroent.* 14, suppl. 53, 9-13, 1979
- Sundler, F., Håkanson, R. and Larsson, L.-I.: Ontogeny of rat pancreatic polypeptide (PP) cells. *Cell Tissue Res.* 178, 303-306, 1977a
- Sundler, F., Håkanson, R., Larsson, L.-I., Brodin, E. and Nilsson, G.: Substance P in the gut: An immunochemical and immunohistochemical study of its distribution and development. In: *Substance P*. Eds. S. von Euler and B. Pernow, Raven Press, New York, 1977b, pp. 59-65

- Sundler, F., Håkanson, R. and Leander, S.: Peptidergic nervous systems in the gut. *Clin. Gastroenterology* 9, 517-543, 1980
- Sutherland, E.W. and de Duve, C.: Origin and distribution of the hyperglycemic-glycogenolytic factor of the pancreas. *J. Biol. Chem.* 175, 663-674, 1948
- Swartzendruber, D.C. and Richter, C.B.: Mucous and argentaffin cells in colonic adenocarcinomas of tamarins and rats. *Lab. Invest.* 43, 523-529, 1980
- Tager, H., Hohenboken, M., Markese, J. and Dinerstein, R.J.: Identification and localization of glucagon-related peptides in rat brain. *Proc. Natl. Acad. Sci. USA* 77, 6229-6233, 1980
- Terenius, L.: Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta pharmacol. et toxicol.* 32, 317-320, 1973
- Track, N.S., Creutzfeldt, C., Litzenberger, J., Neuhoﬀ, C., Arnold, R. and Creutzfeldt, W.: Appearance of gastrin and somatostatin in the human fetal stomach, duodenum and pancreas. *Digestion* 19, 292-306, 1979
- Unger, R.H., Eisentraut, A., Sims, K., McCall, M.S. and Madison, L.L. : Sites of origin of glucagon in dogs and humans. *Clin. Res.* 9, 53, 1961
- Unger, R.H., Ketterer, H. and Eisentraut, A.M.: Distribution of immunoassayable glucagon in gastrointestinal tissues. *Metabolism* 15, 865-867, 1966
- Unger, R.H. and Orci, L.: Physiology and Pathophysiology of glucagon. *Physiol. Rev.* 56, 778-826, 1976
- Van Noorden, S. and Falkmer, S.: Gut - islet endocrinology - some evolutionary aspects. *Invest. Cell Pathol.* 3, 21-35, 1980
- Vassallo, G., Solcia, E. and Capella, C.: Light and electron microscopic identification of several types of endocrine cells in the gastrointestinal mucosa of the cat. *Z. Zellforsch.* 98- 333-356, 1969
- Wilander, E., Grimelius, L., Lundqvist, G. and Skoog, V.: Polypeptide hormones in argentaffin and argyrophil gastroduodenal endocrine tumors. *Am. J. Pathol.* 96, 519-530, 1979a
- Wilander, E., Grimelius, L., Portela-Gomes, G., Lundqvist, G., Skoog, V. and Westermark, P.: Substance P and enteroglucagon-like immunoreactivity in argentaffin and argyrophil midgut carcinoid tumours. *Scand. J. Gastroent.* 14, Suppl. 53, 19-25, 1979b
- Wilander, E., Portela-Gomes, G., Grimelius, L., Lundqvist, G. and Skoog, V.: Enteroglucagon and substance P-like immunoreactivity in argentaffin and argyrophil rectal carcinoids. *Virchows Arch. B Cell Path.* 25, 117-124, 1977
- Zollinger, R.M., Ellison, E.C., Fabri, P.J., Johnson, J., Sparks, J. and Carey, L.C.: Primary peptic ulcerations of the jejunum associated with islet cell tumors. Twenty-five-year appraisal. *Ann. Surg.* 192, 422-427, 1980

Ultrastructural Identification of Cells Storing Pancreatic-Type Glucagon in Dog Stomach

F. Sundler¹, J. Alumets¹, J. Holst², L.-I. Larsson³, and R. Håkanson⁴

Departments of Histology¹ and Pharmacology⁴, University of Lund, Lund, Sweden,
Department of Clinical Chemistry, Division of Gastrointestinal Endocrinology²,
Bispebjerg Hospital, Copenhagen, Denmark and Institute of Medical Biochemistry³,
University of Århus, Denmark

Summary. The oxyntic mucosa of the dog stomach is rich in cells storing pancreatic-type glucagon. The cells were identified by immunocytochemistry and found to be indistinguishable from pancreatic A cells in the electron microscope. Ultrastructural identification of the immunoreactive cells was accomplished by the use of consecutive semithin-ultrathin sections, a technique that permitted the use of optimally fixed material.

Introduction

Pancreatic-type glucagon is present in the gastrointestinal tract of several species (Larsson, Holst, Håkanson and Sundler, 1975; Sasaki, Rubalcava, Baetens, Blazquez, Srikant, Orci and Unger, 1975). The oxyntic mucosa of the dog stomach, which is a particularly rich source of the hormone, seems to contain as much glucagon as does the pancreas (Larsson et al., 1975). In the present report we show that pancreatic-type glucagon in the stomach is stored in cells ultrastructurally indistinguishable from pancreatic A cells.

Materials and Methods

Tissue material was obtained from three adult mongrel dogs. Immediately after sacrifice (nembutal overdose), small pieces of oxyntic mucosa and pancreas were dissected out and immersed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. After fixation overnight the specimens were postfixed in 1% OsO₄ for 1 h, dehydrated in graded ethanol solutions, contrasted with 1% phosphotungstic acid and 0.5% uranyl acetate in ethanol and embedded in Araldite. Identification in the electron microscope of glucagon immunoreactive cells was performed as follows: The plastic was removed from semithin sections (0.1–0.5 µ) by sodium methoxide according to Mayor, Hampton and Rosario (1961). After etching in 1% periodic acid for 5 to 10 min in order to remove osmium (see Beauvillain, Tramu and Dubois, 1975) the sections were processed according to previously reported methods for demonstrating pancreatic-type glucagon (Larsson et al., 1975). An antiserum (No. 4316; Holst and Aasted, 1974) which reacts with pancreatic-type glucagon but not with gut-type glucagon was used in dilution 1:10. The site of antigen-antibody reaction in the sections

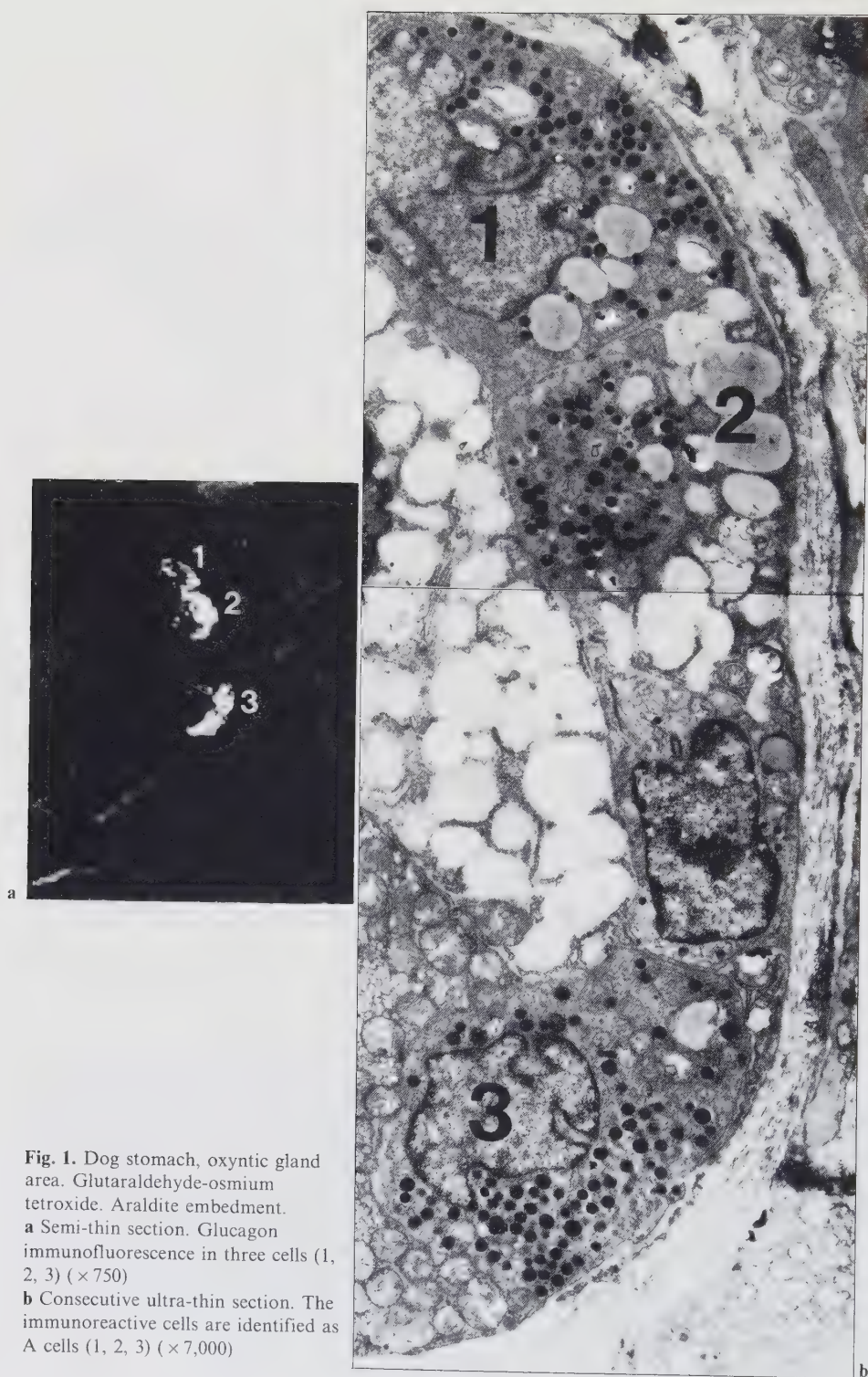


Fig. 1. Dog stomach, oxyntic gland area. Glutaraldehyde-osmium tetroxide. Araldite embedment.
a Semi-thin section. Glucagon immunofluorescence in three cells (1, 2, 3) ($\times 750$)
b Consecutive ultra-thin section. The immunoreactive cells are identified as A cells (1, 2, 3) ($\times 7,000$)

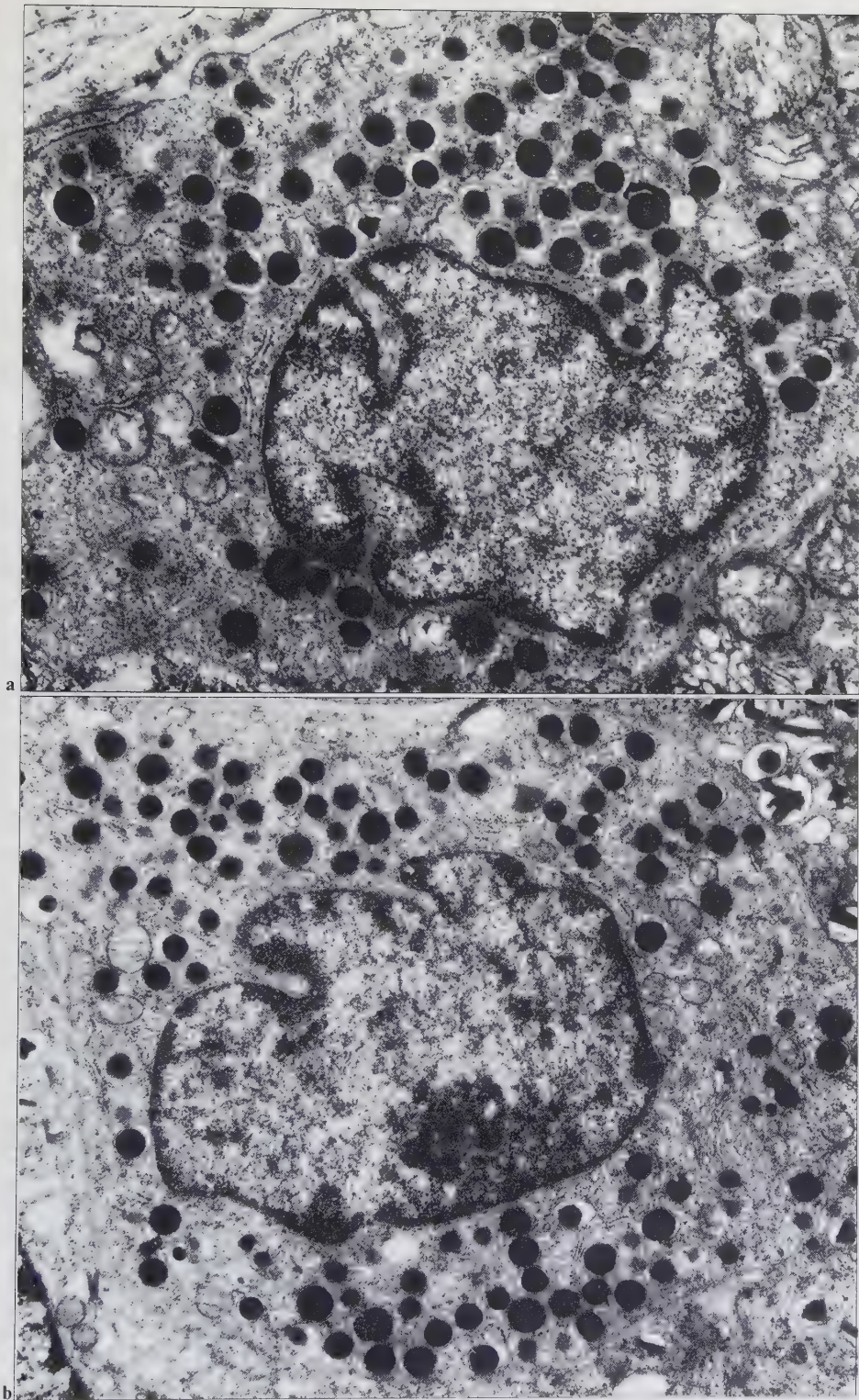


Fig. 2. a Higher magnification of cell no. 3 in Figure 1. Cytoplasmic granules indistinguishable from those of a pancreatic A cell **b** ($\times 17,000$)

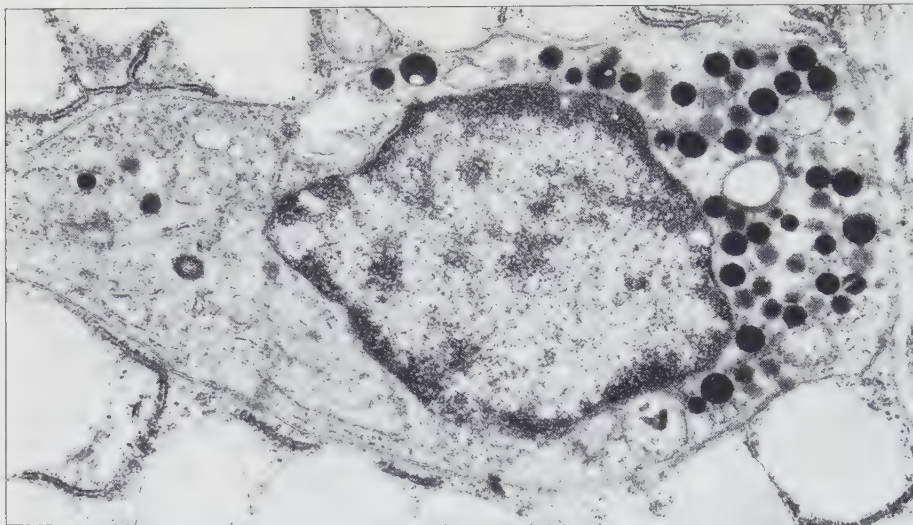


Fig. 3. A-like cell in dog oxyntic mucosa ($\times 17,000$)

was revealed by fluorescein isothiocyanate-labelled sheep anti-rabbit IgG diluted 1:20. The sections were examined in a Leitz Orthoplan fluorescence microscope equipped with an epi-illumination system. Standard filter setting No. 3 (peak excitation at 490 nm) was used. Immediately adjacent ultrathin sections were stained with lead citrate and uranyl acetate and examined in a Philips EM 300 electron microscope. By this procedure one and the same cell was observed in the fluorescence as well as in the electron microscope. As controls served sections treated with glucagon antiserum inactivated by the addition of 20 μ g of porcine pancreatic glucagon per ml diluted serum.

Results

Numerous glucagon immunoreactive cells were observed in the oxyntic mucosa, predominantly in the basal half of the glands (Fig. 1a). Controls were negative. The immunoreactive cells contained numerous round, membrane-bound cytoplasmic granules of moderate to high electron density (Fig. 1b). Usually the dense core of the granules was separated from the limiting membrane by a distinct electron-lucent rim (Fig. 2a). The cells seemed to lack luminal contact. The ultrastructural properties of the gastric glucagon cell were indistinguishable from those of the pancreatic A cells (Fig. 2b).

In addition to the glucagon immunoreactive cells the oxyntic mucosa harboured a number of other endocrine cell types, including ECL cells, D cells and one cell type that had cytoplasmic granules somewhat resembling those of the glucagon cells (Fig. 3). The granules of this cell type were round, membrane-bound and very electron dense but usually lacked a distinct electron-lucent rim between the dense core and the surrounding membrane. On the basis of the morphology of their granules these cells (labelled X cells by Solcia, Capella, Vassallo and Buffa, 1975) seem to be equivalent to the A-like cells which have been described in the oxyntic mucosa of other species (cf. Solcia, Pearse, Grube, Kobayashi, Bussolati, Creutzfeldt and Gepts, 1973). They were about as numerous as the glucagon cells.

Discussion

Earlier reports have emphasized the existence in the dog stomach of cells with ultrastructural and tinctorial properties indistinguishable from those of pancreatic A cells (Kubes, Jirasek and Lomsky, 1974; Sasaki et al., 1975; Solcia et al., 1975; Baetens, Rufener, Srikant, Dobbs, Unger and Orci, 1976a; Grimelius, Capella, Buffa, Polak, Pearse and Solcia, 1976). Using the technique of consecutive semithin-ultrathin sections from conventionally fixed material we now demonstrate that the glucagon immunoreactive cells in the dog stomach are identical with A cells.

Baetens et al. (1976a, b) used antiperoxidase (PAP)-labeling at the electron microscopic level and claimed that the gastric glucagon cells were A cells. However, this procedure, which as a rule does not permit the use of optimally fixed material, precludes examination of fine structural details and hence hampers identification of the cell. Both Grimelius et al. (1976) and Baetens et al. (1976a) argued that the gastric A cell is glucagon-producing on the basis of similarities in histological and ultrastructural properties with the pancreatic A cell. This assumption has now proved correct.

Acknowledgements. Grant support from Swedish Medical Research Council (No. 04X-04499), Riksföreningen mot Cancer (806-B75-02X) and Albert Pålsson's Foundation.

References

- Baetens, D., Rufener, C., Srikant, B.C., Dobbs, R., Unger, R., Orci, L.: Identification of glucagon-producing cells (A cells) in dog gastric mucosa. *J. Cell Biol.* **69**, 455-464 (1976a)
- Baetens, D., Rufener, C., Unger, R.H., Renold, A.E., Orci, L.: Identification par immunocytochimie de la cellule sécrétant le glucagon dans la muqueuse gastrique. *C.R. Acad. Sci. (Paris)* **282**, 195-197 (1976b)
- Beauvillain, J.-C., Tramu, G., Dubois, M.P.: Characterization by different techniques of adrenocorticotropin and gonadotropin producing cells in Lerot pituitary (*Eliomys quercinus*). *Cell Tiss. Res.* **158**, 301-317 (1975)
- Grimelius, L., Capella, C., Buffa, R., Polak, J.M., Pearse, A.G.E., Solcia, E.: Cytochemical and ultrastructural differentiation of enteroglucagon and pancreatic-type glucagon cells of the gastrointestinal tract. *Virchows Arch. Abt. B Cell Path.* **20**, 217-228 (1976)
- Holst, J., Aasted, B.: Production and evaluation of glucagon antibodies for radioimmunoassay. *Acta endocr. (Kbh.)* **77**, 715-726 (1974)
- Kubes, L., Jirasek, K., Lomsky, R.: Endocrine cells of the dog gastrointestinal mucosa. *Cytologia (Tokyo)* **39**, 179-194 (1974)
- Larsson, L.-I., Holst, J., Håkanson, R., Sundler, F.: Distribution of glucagon immunoreactivity in the digestive tract of various mammals: an immunohistochemical and immunochemical study. *Histochemistry* **44**, 281-290 (1975)
- Mayor, H.D., Hampton, J.C., Rosario, R.: A simple method for removing the resin from epoxy-embedded tissue. *J. biophys. Cytol.* **9**, 909-910 (1961)
- Sasaki, H., Rubalcava, B., Baetens, D., Blazquez, E., Srikant, C.B., Orci, L., Unger, R.H.: Identification of glucagon in the gastrointestinal tract. *J. clin. Invest.* **56**, 135-145 (1975)
- Solcia, E., Capella, C., Vassallo, G., Buffa, R.: Endocrine cells of the gastric mucosa. *Int. Rev. Cytol.* **42**, 223-286 (1975)
- Solcia, E., Pearse, A.G.E., Grube, D., Kobayashi, S., Bussolati, G., Creutzfeldt, W., Gepts, W.: Revised Wiesbaden classification of gut endocrine cells. *Rendic. Gastroent.* **5**, 13-16 (1973)

Distribution, Ontogeny and Ultrastructure of the Mammalian Secretin Cell*

L.-I. Larsson, F. Sundler, J. Alumets, R. Håkanson,
O.B. Schaffalitzky de Muckadell, and J. Fahrenkrug

Institute of Medical Biochemistry, University of Aarhus, Aarhus, Denmark;
Departments of Histology and Pharmacology, University of Lund, Lund, Sweden;
and Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark

Summary. Immunocytochemically, secretin cells have been demonstrated to occur in the duodenum and jejunum of several mammals. Calculations on the relative frequency of such cells indicate that the bulk of secretin occurs in the jejunum, a fact supporting the view that secretin may be released by physiological stimulants other than hydrochloric acid. Electron microscopical identification of cat and pig secretin cells confirmed their identity with the ultrastructurally defined S cells, and staining experiments revealed that secretin cells were argyrophilic both with the method of Grimelius and with that of Hellerström and Hellman. Secretin cells are detected already in the 17-day old fetal rat duodenum and show a developmental pattern similar to that displayed by the gastrin cells. It is suggested that secretin may play a role in the early regulation of growth of the fetal gastrointestinal tract.

Key words: Mammalian secretin cell — Distribution — Ontogeny — Ultrastructure — Immunocytochemistry.

Introduction

The mucosa of the gastrointestinal tract contains many different types of hormone-producing cells. A number of biologically active peptides have been isolated from the antrum and the upper portion of the small intestine (Gregory, 1974; Grossman, 1974). An exact localization of these peptides is important for a full understanding of the regulation of gut function. Identification of gastrointestinal endocrine cells is often attempted by comparing the distribution of cells demonstrated with more or less selective stains or electron microscopy with the distribution of the hormone as demonstrated by bioassay or immunological techniques.

Send offprint requests to: Lars-Inge Larsson, M.D., Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

* *Acknowledgements:* Supported by grants from the Danish and Swedish MRC

At the beginning of this century the first gastrointestinal hormone, secretin, was discovered (Bayliss and Starling, 1902; Starling, 1905). Secretin cells, demonstrated by immunocytochemistry (Polak et al., 1971; Bussolati et al., 1971; Solcia et al., 1972; Chey and Escoffery, 1976), have been suggested to be identical with the ultrastructurally defined S cells (Polak et al., 1971; Bussolati et al., 1971; Solcia et al., 1972). The diversity of endocrine cell types in the gut (Solcia et al., 1973; Solcia et al., 1974), however, makes such a tentative identification uncertain. Due to technical improvements, possibilities now exist for examining ultrastructural, tinctorial and immunocytochemical properties of one and the same cell. These methods were therefore chosen to study the mammalian secretin cell in the present work. In addition, information concerning the distribution and ontogeny of the secretin cells is given.

Material and Methods

Tissue Material. Adult rats, cats and dogs of both sexes were killed with diethyl ether (rats) or mebumal (Nembutal®). Material from pigs was collected at a nearby abattoir and human surgical specimens (carcinoma or ulcer cases) were kindly provided by Drs. Stig Ingemansson and Gustav Liedberg, Department of Surgery, Hospital of Lund, Sweden. In addition, duodenal tissue specimens were collected from rat fetuses from the 17th gestational day onwards and from newborn and young rats (1–14 days). From adult animals and man, specimens were taken from various locations along the gastrointestinal tract. The specimens were freeze-dried and vapor-fixed with either formaldehyde (Björklund et al., 1972) or diethylpyrocarbonate (DEPC) (Pearse and Polak, 1975), embedded in paraffin in vacuo and sectioned at 5 µm. In addition, small pieces of cat and pig duodenal mucosa were fixed overnight in 1% glutaraldehyde and 3% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.1. Part of these specimens were postfixed for 1 h in 1% osmium tetroxide. All specimens were stained with 1% phosphotungstic acid and 0.5% uranyl acetate and embedded in Epon 812.

Antisera. Secretin antiserum No. 5585-3 was used throughout. It shows high specificity, avidity and binding capacity to secretin. Details on specificity, avidity and titer are given elsewhere (Fahrenkrug et al., 1976). Fluorescein isothiocyanate-labeled and unlabeled goat anti-rabbit IgG were purchased from SBL, Stockholm, Sweden. The peroxidase-antiperoxidase (PAP) complex was obtained from Dakopatts, Copenhagen, Denmark.

Immunocytochemistry. Deparaffinized or deplasticized (Mayor, Hampton and Rosario, 1961) sections were carried down to water and subjected to an indirect immunocytochemical method for the demonstration of secretin. Antiserum No. 5585-3 was used as first layer and the site of antigen-antibody reaction was revealed either by fluorescein isothiocyanate-labeled antirabbit IgG or by the PAP technique of Sternberger (1974). The first antiserum was used in dilution 1:80 (applied for 30 min; immunofluorescence) or 1:20,000 (applied for 24 h; PAP technique).

Controls were run as recommended by Sternberger (1974) and included the application of antiserum pretreated with secretin [33 pmol synthetic porcine secretin (kindly donated by Prof. E. Wünsch, Munich, FRG) per ml antiserum diluted 1:80].

The sections were examined either in a Leitz Orthoplan fluorescence microscope equipped with an epiillumination system or in a similarly equipped Zeiss standard microscope. A Xenon XBO 75 burner served as light source and filter settings were selected to give peak excitation at 490 nm.

Conventional Stains. Sections treated for immunofluorescence were occasionally restained with silver according to Grimelius (1968) or Hellerström and Hellman (1960) (argyrophilic stains).

Electron Microscopy. Formaldehyde-glutaraldehyde fixation preserved secretin immunoreactivity and provided acceptable ultramorphology. Post-osmification gave considerable improvement of the ultrastructure but rendered demonstration of immunoreactivity impossible. Removal of osmium

from the sections by treatment with 1% periodic acid for 10 min, however, restored the immunoreactivity (cf. Beauvillain et al., 1975). This procedure enabled the use of optimally treated specimens throughout the study. For identification of secretin cells, ultrathin sections, stained with lead citrate and uranyl acetate, were compared with adjacent semithin sections treated for immunocytochemical demonstration of secretin. Granular size was established by measuring the diameter of all cytoplasmic granules in seven secretin cells from cats and two from pigs. All cells used for establishing granule size had been identified by the consecutive semithin/ultrathin section technique.

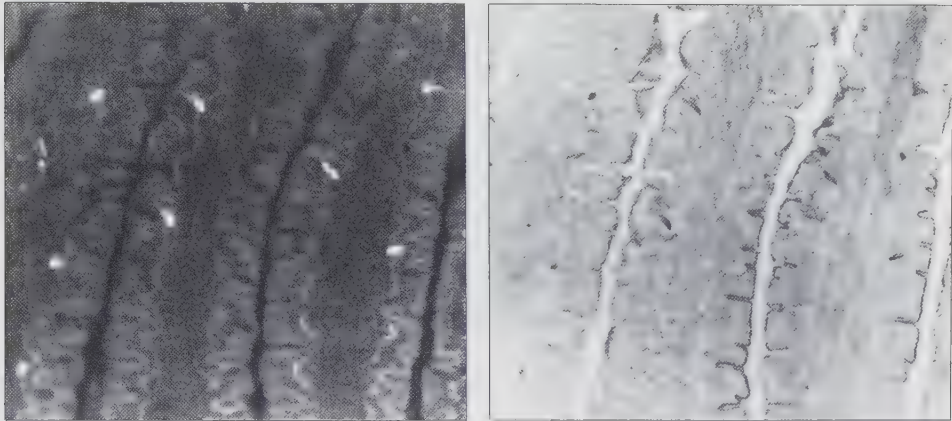


Fig. 1. Cat duodenal mucosa. Left: Secretin immunofluorescence. Numerous secretin cells are present in the epithelium of the villi. Right: Restaining of the same section with the silver impregnation technique of Grimelius. The secretin cells are argyrophilic. $\times 195$

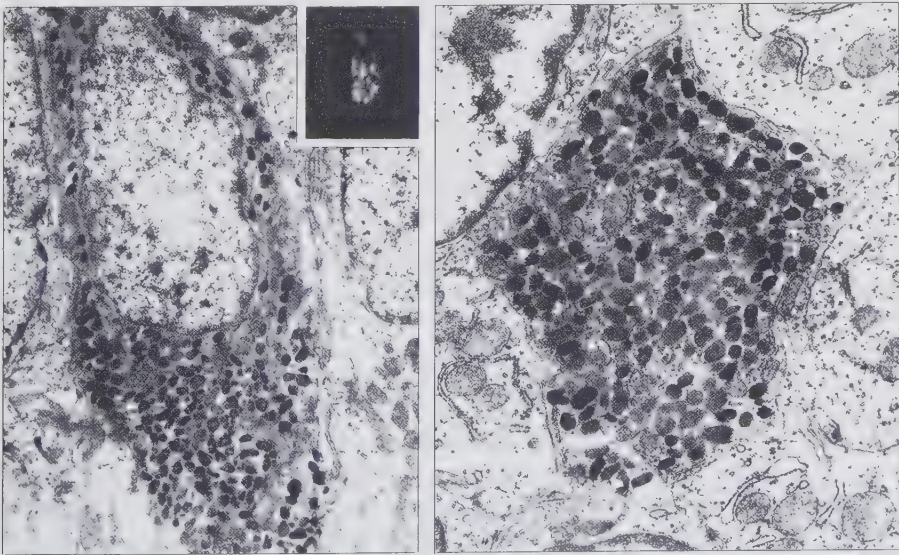


Fig. 2. Cat duodenum. Left: Secretin cell identified by immunofluorescence performed on the adjacent semithin section (insert, $\times 720$). Right: Higher magnification of secretin cell process (identified as above) to show the pleomorphism of the secretory granules. Left, $\times 7420$; right, $\times 12,250$

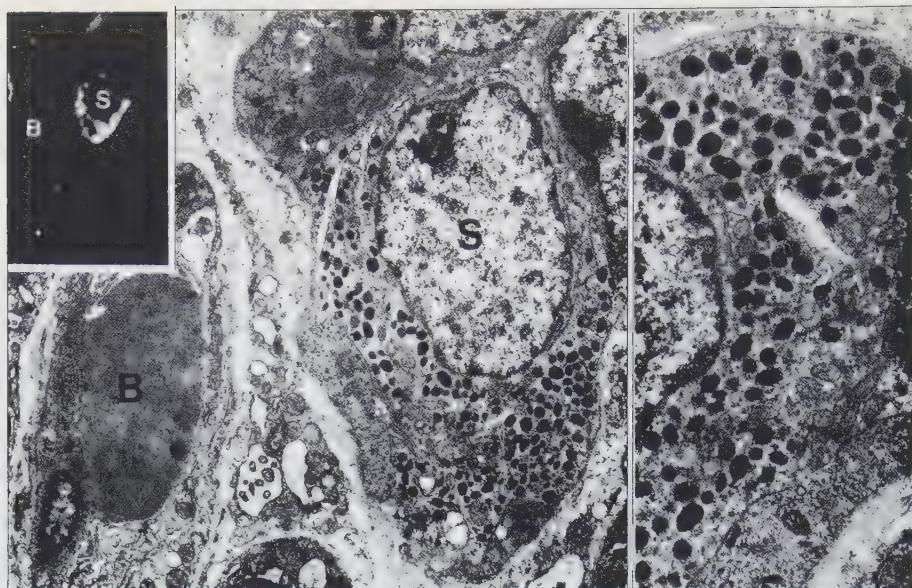


Fig. 3. Pig duodenum. Left: Secretin cell identified by immunofluorescence on adjacent semithin section (insert, $\times 765$). For orientation: *B* blood vessel, *S* secretin cell. Right: Higher magnification of the same cell. Left, $\times 7280$; right, $\times 12,250$

Results

Secretin cells were detected immunocytochemically in the small intestines of all species studied. In all species, except the rat, both formaldehyde and DEPC fixation preserved secretin immunoreactivity. In the rat, however, secretin cells could be clearly demonstrated only following DEPC fixation. In all species studied secretin cells predominated in the duodenal and jejunal mucosa. In specimens from midjejunal mucosa they appeared to be about half to one-third as numerous per unit area as in the mid-portion of the duodenum. They were rarely seen in antral and ileal mucosa and never in the remainder of the gastrointestinal tract. In the duodenum and jejunum they usually occurred in the epithelium on the villi and in the zones of transition between villi and crypts (Fig. 1a). Secretin cells were very numerous in the cat, fewer in the dog, pig and man and very few in the rat. Cats and pigs were chosen for the further characterization of secretin cells.

The secretin cells stained strongly with silver using the procedure of Grimelius (Fig. 1a, b) but weakly with the procedure of Hellerström and Hellman.

At the ultrastructural level feline and porcine secretin cells were characterized by the presence of numerous electron dense granules in their cytoplasm (Figs. 2, 3). The granules were often densely accumulated in the basal part of the cells. The granules were membrane-bounded, sometimes with a narrow electron lucent rim separating their membranes from the dense core. They were characteristically pleomorphic. Often ovoid, discoid or rod-shaped profiles were encountered, thus somewhat resembling those of enterochromaffin cell granules. The

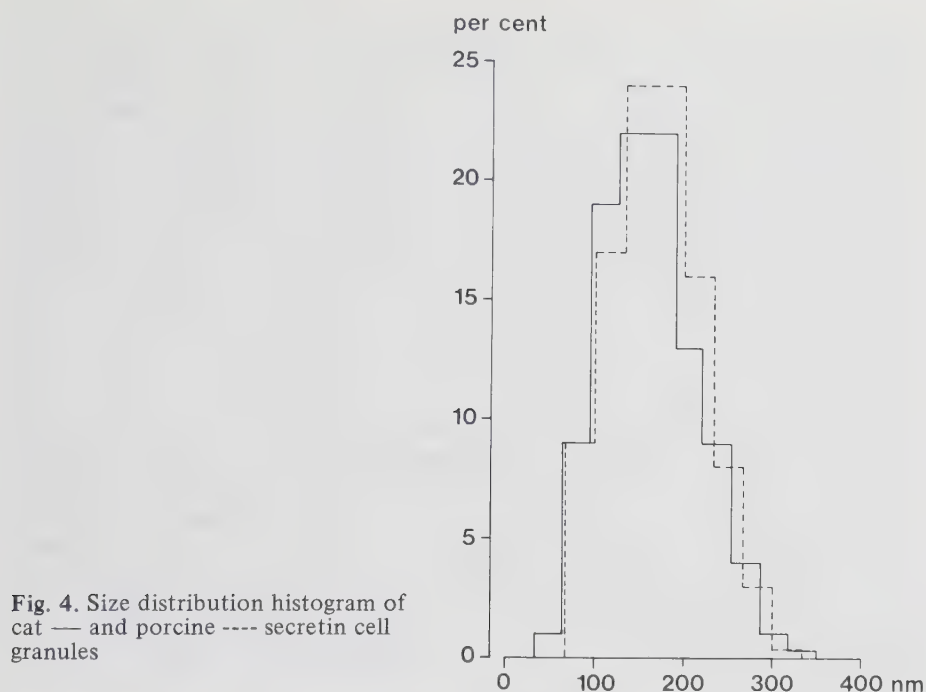


Fig. 4. Size distribution histogram of cat — and porcine ---- secretin cell granules

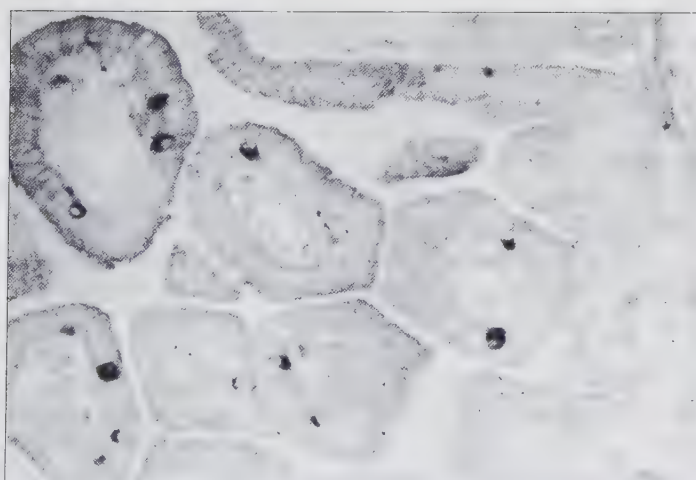


Fig. 5. Duodenal mucosa from 20-day old rat fetus, stained with the secretin-PAP method. Numerous strongly immunoreactive secretin cells are seen. $\times 300$

mean granule diameter in cat secretin cells was 180 ± 55 (S.D.) nm ($n = 1145$). The corresponding value for the porcine secretin cells was 186 ± 52 (S.D.) nm ($n = 271$). Size distribution histograms of cat secretin cell granules are presented in Figure 4. Occasionally the secretin cells were seen to reach the lumen via a narrow neck, the luminal surface being endowed with microvilli.

The development of duodenal secretin immunoreactive cells was studied in fetal and new-born rats. Secretin cells were present in the duodenum at a fetal age of 17 days and increased gradually in number up to the 4th postnatal day (Fig. 5). It should be noted that at the earliest stages studied (17–18 day old fetuses) the PAP technique was essential for demonstrating the secretin cells. Interestingly, in fetuses 18–21 days old and in young rats they were considerably more numerous per unit area than in the adult animals. Four days after birth their number per unit area started to decrease and a near-adult frequency of occurrence was established at about the 14th day.

Discussion

The distribution of secretin cells agrees well with results obtained by bioassay (Bayliss and Starling, 1902; Starling, 1905; Krawitt et al., 1966) and immunoassay (Fahrenkrug et al., 1976). In contrast to the vasoactive intestinal polypeptide (VIP), which in the intestines has been shown to be exclusively localized in a rich system of gastrointestinal nerves (Larsson et al., 1976), secretin immunoreactivity was found exclusively in endocrine cells of the intestinal epithelium. These findings confirm earlier observations made by Bayliss and Starling (1902) showing secretin activity to occur in the epithelium while the “specific chemical vasodilator”, which probably in part accounts for VIP, occurred in deeper layers of the gut wall.

The secretin cells stained with silver impregnations known to demonstrate the cytoplasmic granules of endocrine cells. At the ultrastructural level the secretin cells demonstrate very distinct granules. Their proposed identity with the S cells (Polak et al., 1971; Bussolati et al., 1971; Solcia et al., 1972) has thus been confirmed. Secretin cells were sometimes seen to reach the intestinal lumen via a neck covered with microvilli. Thus, the secretin cells belong to the open type of endocrine cells (Fujita and Kobayashi, 1974). This would indicate that the luminal content may stimulate the release of secretin. A somewhat unexpected finding was the staining of the secretin cells with the silver method of Hellerström and Hellman (1960), known to demonstrate pancreatic and gastrointestinal A₁ (or D) cells. Hence, caution must be exercised when interpreting gastrointestinal endocrine cells and tumour cells as A₁ cells on the basis of silver staining alone.

In the species studied, secretin cells were most numerous in the duodenal and jejunal epithelium. Recently Chey and Escoffery (1976) reported on the occurrence of numerous secretin immunoreactive cells in the antral mucosa of man, dog and rat. In the present study secretin cells were only rarely seen in the antral mucosa. Thus, on this point our data as well as those of others (Polak et al., 1971, Solcia et al., 1972) do not agree in full with the findings of Chey and Escoffery. At present, this discrepancy remains unexplained.

Interestingly, quite a few secretin cells were found in the jejunum (averaging half to one-third of the frequency of occurrence of duodenal secretin cells). It has been argued that the low pH necessary for release of secretin may, if at all, only occur in the most proximal portion of the duodenum (Thomas, 1950; Wormsley, 1973; Fahrenkrug et al., 1977). Assuming a constant frequency of secretin cells

in the jejunum (as calculated from their frequency of occurrence in mid-jejunum) the bulk of intestinal secretin is surely found in the jejunum. This view is further corroborated by the findings of Bayliss and Starling (1902) of substantial quantities of jejunal secretin bioactivity. The pH of the jejunal contents is unlikely to reach the values reported to be necessary for release of secretin (Grossman, 1974). While it cannot be excluded that secretin present in the proximal small intestine may be released by low pH, this mechanism does not seem valid for the bulk of intestinal secretin. Conceivably other release mechanisms may exist, perhaps reflecting other physiological functions of secretin. Recently Johnson (1976) made the provocative suggestion that the trophic effects of the gastrointestinal hormones may be their main physiologic action. Gastrin has been shown to exert trophic effects on a number of gastrointestinal tissues, including the parietal cells (for references see Johnson, 1976). This effect appears to be counteracted by secretin (Johnson and Guthrie, 1974), which thus may be an "anti-trophic" factor. In support of the alleged role of gastrin in the development of the gastrointestinal tract numerous gastrin cells are found at early stages of development of this organ (Larsson et al., 1975; Larsson et al., 1974, 1976). In the rat, gastrin cells are present in the duodenum at a fetal age of 17 days and increase progressively in number until a few days after birth when their number (per unit area) starts to decrease (Larsson et al., 1976, and unpublished observations). Thus, duodenal secretin cells and gastrin cells appear to show a parallel development. It seems possible therefore that gastrin and secretin may act in conjunction to regulate the development of gastrointestinal tissues.

References

- Bayliss, W.M., Starling, E.H.: The mechanism of pancreatic secretion. *J. Physiol. (Lond.)* **28**, 325–353 (1902)
- Beauvillain, J.-C., Tramu, G., Dubois, M.P.: Characterization by different techniques of adrenocorticotropin and gonadotropin producing cells in Lerot pituitary (*Eliomys quercinus*). *Cell Tiss. Res.* **158**, 301–317 (1975)
- Björklund, A., Falck, B., Owman, C.: Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In: *Methods of investigative and diagnostic endocrinology*, Vol. 1 (S.A. Berson, ed.), pp. 318–368. Amsterdam: North-Holland Publishing Co. 1972
- Bussolati, G., Capella, C., Solcia, E., Vasallo, G., Vezzadini, P.: Ultrastructural and immunofluorescent investigations on the secretin cell in the dog intestinal mucosa. *Histochemie* **26**, 218–227 (1971)
- Chey, W.Y., Escoffery, R.: Secretin cells in the gastrointestinal tract. *Endocrinol.* **98**, 1390–1395 (1976)
- Fahrenkrug, J., Schaffalitzky de Muckadell, O.B., Holst, J.J.: Plasma secretin concentration in anaesthetized pigs after intraduodenal glucose, fat, amino acids or meals with various pH. *Scand. J. Gastroent.* (in press) (1977)
- Fahrenkrug, J., Schaffalitzky de Muckadell, O.B., Rehfeld, J.F.: Production and evaluation of antibodies for radioimmunoassay of secretin. *Scand. J. clin. Lab. Invest.* **36**, 281–287 (1976)
- Fujita, T., Kobayashi, S.: The cells and hormones of the GEP endocrine system. In: *Gastro-entero-pancreatic endocrine system* (T. Fujita, ed.), pp. 1–17. Stuttgart: G. Thieme 1974
- Gregory, R.A.: The gastrointestinal hormones: A review of recent advances (The Bayliss-Starling Lecture 1974). *J. Physiol. (Lond.)* **24**, 1–3 (1974)
- Grimelius, L.: A silver nitrate stain for α_2 -cells in human pancreatic islets. *Acta Soc. Med. upsalien.* **73**, 243–270 (1968)

- Grossman, M.I.: Candidate hormones of the gut. *Gastroenterol.* **67**, 730–755 (1974)
- Grossman, M.I., Konturek, S.J.: Gastric acid does drive pancreatic bicarbonate secretion. *Scand. J. Gastroent.* **9**, 299–302 (1974)
- Hellerström, C., Hellman, B.: Some aspects of silver impregnation of the islets of Langerhans in the rat. *Acta endocr. (Kbh.)* **35**, 518–532 (1960)
- Johnson, L.R.: The trophic action of gastrointestinal hormones. *Gastroenterol.* **70**, 278–288 (1976)
- Johnson, L.R., Guthrie, P.D.: Secretin inhibition of gastrin-stimulated deoxyribonucleic acid synthesis. *Gastroenterol.* **67**, 601–606 (1974)
- Krawitt, E.L., Zimmerman, G.R., Clifton, J.A.: Location of secretin in dog duodenal mucosa. *Amer. J. Physiol.* **211**, 935–938 (1966)
- Larsson, L.-I., Fahrenkrug, J., Schaffalitzky de Muckadell, O., Sundler, F., Håkanson, R., Rehfeld, J.F.: Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. *Proc. nat. Acad. Sci. (Wash.)* **73**, 3197–3200 (1976)
- Larsson, L.-I., Håkanson, R., Sjöberg, N.-O., Sundler, F.: Fluorescence histochemistry of the gastrin cell in fetal and adult man. *Gastroenterol.* **68**, 1152–1159 (1975)
- Larsson, L.-I., Rehfeld, J.F., Sundler, F., Håkanson, R.: Pancreatic gastrin in foetal and neonatal rats. *Nature (Lond.)* **262**, 609–610 (1976)
- Larsson, L.-I., Rehfeld, J.F., Sundler, R., Håkanson, R., Stadil, F.: Concomitant development of gastrin immunoreactivity and formaldehyde-ozone-induced fluorescence in gastrin cells of rabbit antropyloric mucosa. *Cell Tiss. Res.* **149**, 329–332 (1974)
- Mayor, H.D., Hampton, J.C., Rosario, B.: A simple method for removing the resin from epoxy-embedded tissue. *J. biophys. biochem. Cytol.* **9**, 909–910 (1961)
- Pearse, A.G.E., Polak, J.M.: Bifunctional reagents as vapour- and liquid-phase fixatives for immunohistochemistry. *Histochem. J.* **7**, 179–186 (1975)
- Polak, J.M., Bloom, S., Coulling, I., Pearse, A.G.E.: Immunofluorescent localization of secretin in the canine duodenum. *Gut* **12**, 605–610 (1971)
- Solcia, E., Capella, C., Buffa, R., Bettini, R.: The endocrine cells of the gastrointestinal mucosa. *Endocrinol.* 1973. Heinemann Medical Books, Ltd. 1974
- Solcia, E., Capella, C., Vezzadini, P., Barbara, L., Bussolati, G.: Immunohistochemical and ultrastructural detection of the secretin cell in pig intestinal mucosa. *Experientia (Basel)* **28**, 549–550 (1972)
- Solcia, E., Pearse, A.G.E., Grube, S., Kobayashi, G., Bussolati, G., Creutzfeldt, W., Gepts, W.: Revised Wiesbaden classification of gut endocrine cells. *Rendic. Gastroenterol.* **5**, 13–16 (1973)
- Starling, E.H.: The chemical correlation of the functions of the body. *Lancet* **1905 II**, 339–341
- Sternberger, L.A.: Immunocytochemistry. New Jersey: Prentice-Hall Inc. 1974
- Thomas, J.E.: The external secretion of the pancreas. Springfield, Illinois: Charles C. Thomas Co. 1950
- Wormsley, K.G.: Is secretin secreted? *Gut* **14**, 743–751 (1973)

Accepted March 30, 1977

Immunohistochemical Localization of Neurotensin in Endocrine Cells of the Gut

F. Sundler¹, R. Håkanson², R.A. Hammer³, J. Alumets¹, R. Carraway³, S.E. Leeman³, and E.A. Zimmerman⁴

Departments of Histology¹ and Pharmacology², University of Lund, Lund, Sweden, Department of Physiology and Laboratory of Human Reproduction and Reproductive Biology³, Harvard Medical School, Boston, Mass., USA, and Department of Neurology⁴, Columbia University, College of Physicians and Surgeons, New York, New York, USA

Summary. Endocrine cells displaying neurotensin immunoreactivity are found scattered in the jejuno-ileum of all mammals studied, including man. They are rather scarce in rat, guinea pig, rabbit and pig and fairly numerous in cat, dog and man. In most mammals the neurotensin cells predominate on the villi. Only in the dog are they more numerous in the crypts. In the chicken, neurotensin cells occur all along the intestinal tract. They are particularly numerous in the zone that joins the gizzard with the duodenum. The ontogeny of the neurotensin cells in the gut was studied in rats and chickens. In the rat, the cells are first observed in the jejuno-ileum immediately before birth. The adult frequency is reached 4–5 days later. In the chicken, neurotensin cells first appear in the colon in the 18 day old embryo and in the small intestine two days later (i.e. one or two days before hatching). A few days after hatching, the gut has achieved the adult number of neurotensin cells per unit area.

Key words: Endocrine cells — Gut — Neurotensin — Immunocytochemistry — Comparative studies.

Introduction

Neurotensin is a tridecapeptide recently isolated from extracts of bovine hypothalami (Carraway and Leeman, 1973). The structure of the peptide has been established (Carraway and Leeman, 1975a), and its biological actions are currently being investigated. In addition to producing hypotension and gut contraction, neurotensin induces cyanosis and hyperglycemia (Carraway, Demers and Leeman, 1976). It also affects the release of ACTH, LH, and FSH when given

Send offprint requests to: Dr. Frank Sundler, Histologiska institutionen, Biskopsgatan 5, S-223 62 Lund, Sweden

to anesthetized rats (Makino et al., 1973). Recent observations also indicate that neurotensin is a powerful blocker of pentagastrin-stimulated gastric acid secretion in the dog (Anderson et al., 1976). The preparation of synthetic neurotensin (Carraway and Leeman, 1975b) has made possible the development of a highly sensitive and specific radioimmunoassay (Carraway and Leeman, 1976a). Using this radioimmunoassay, immunoreactive neurotensin has been demonstrated in the rat and rabbit alimentary canal, the highest concentrations being found in the jejunum-ileum where they are similar to that in the hypothalamus (Carraway and Leeman, 1976b). The structure of immunoreactive neurotensin from calf small intestine has also been found to be very similar if not identical to that of the hypothalamic substance (Kitabgi et al., 1976). In the present paper the localization of immunoreactive neurotensin in endocrine cells of the intestinal epithelium of several species including man is described. In addition, findings on the ontogeny of the neurotensin-containing cells in the gut of the rat and chicken are reported. In view of its potent pharmacological actions and its presence in endocrine cells, it is tempting to speculate that neurotensin is involved in the regulation of gastrointestinal function(s).

Material and Methods

Antiserum against hemocyanin-conjugated synthetic neurotensin was raised in rabbits as previously described (code no. HC-8; Carraway and Leeman, 1976a). Fluorescein isothiocyanate-labeled sheep antiserum against rabbit IgG was obtained from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Goat anti-rabbit IgG serum, used in the immunoperoxidase technique, was obtained from Antibodies, Inc., Davis, California. The peroxidase-antiperoxidase complex (PAP) was provided by L.A. Sternberger, and used according to his technique (1974); it was also purchased from Miles Labs, Elkhart, Indiana. Ten adult Wistar rats and three Sprague-Dawley rats, three rabbits, five guinea pigs, three cats and three dogs were killed by decapitation (rats), injection of air (rabbits), cervical dislocation (guinea pigs), or by an overdose of nembutal (cats and dogs). Material from pigs was obtained from a slaughterhouse. Human material was collected after surgical removal for gastric ulcer or intestinal carcinoma (courtesy of Drs. S. Ingemansson and G. Liedberg, Department of Surgery, University Hospital, Lund, Sweden). Specimens were taken from fetal rats from the 15th day of gestation up to parturition and from new-born and young rats 1–14 days old. The material also included chick embryos (10–22 days of incubation), newly hatched chickens and young chickens 6–10 weeks old. Specimens were taken from the pancreas and from various parts of the gastrointestinal tract, frozen at the temperature of liquid nitrogen in a propane-propylene or isopentane mixture, and freeze-dried. They were then exposed to formaldehyde or diethylpyrocarbonate (DEPC) vapors and embedded in paraffin *in vacuo* (for details, see Larsson et al., 1975). Sections were cut at 5 μ m, deparaffinized in xylene and carried down to water through graded ethanol solutions. They were then exposed for 30 min at room temperature to the neurotensin antiserum in dilution 1:40 (immunofluorescence) and 1:40 or 1:800 (immunoperoxidase staining). Following rinsing in 0.1 M phosphate buffered saline, pH 7.2, the site of antigen-antibody reaction was revealed either by fluorescein-labeled anti-rabbit IgG diluted 1:20 (30 min incubation), or by unlabeled goat anti-rabbit IgG applied at 1:100 dilution for 30 min, followed by a one hour incubation with PAP (1:80 or 1:1000), and five min exposure to 3,3'-diaminobenzidine, 50 mg/100 ml. All solutions used in the immunofluorescence technique contained 0.25% Triton X-100. The sections were rinsed and mounted in phosphate buffered glycerine (immunofluorescence) or dehydrated and mounted in Permount (immunoperoxidase) and examined with a fluorescence or light microscope. Controls included sections exposed to antigen-inactivated antiserum (10 μ g neurotensin per ml diluted antiserum). In one experiment other peptides were added to the antiserum

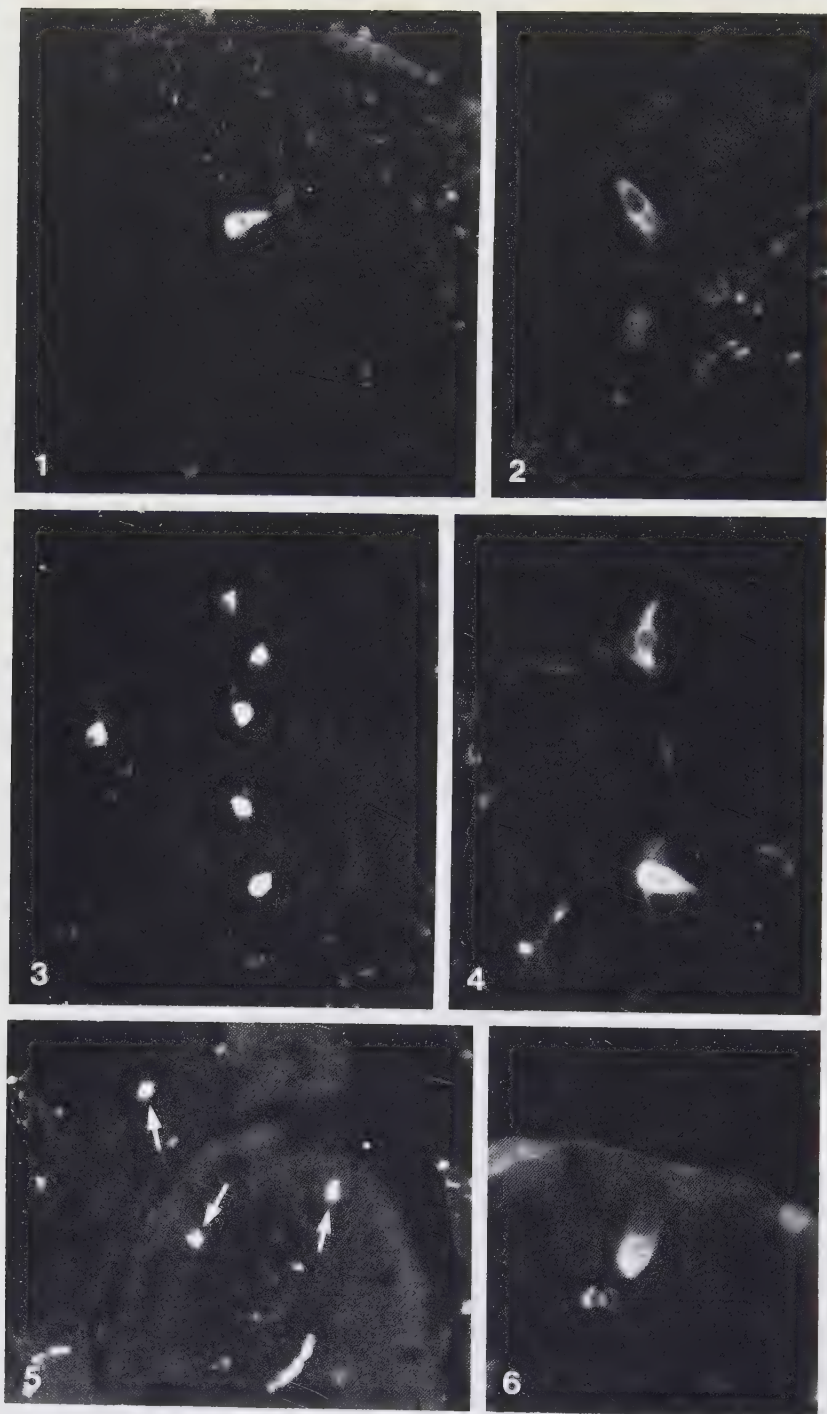
in order to test for signs of inactivation. These peptides included porcine CCK, glucagon, insulin, ACTH, bovine pancreatic polypeptide, synthetic gastrin I, somatostatin, and substance P (in a concentration of 10 μ g peptide per ml of diluted antiserum).

Results

Neurotensin immunoreactivity could be demonstrated in endocrine-like cells in the jejunum and ileum of all mammals examined (Figs. 1-7). Both DEPC and formaldehyde vapor fixation preserved the immunoreactivity although DEPC was superior. Addition of neurotensin to the antiserum blocked the immunoreaction, whereas the other peptides tested did not inhibit the reaction. In the rabbit and pig, neurotensin cells were scarce. They were somewhat more numerous in the rat and guinea pig and still more numerous in the cat, dog and man. In the dog intestine, neurotensin cells were found distributed throughout the mucosa with some predominance in the crypt epithelium, while in the other mammals the cells were usually found on the villi. Table 1 summarizes the topographic distribution and relative frequency of neurotensin cells in the mucosa of the jejunum-ileum of several mammals. The neurotensin cells seemed to be of the "open" type, i.e., they appeared to make luminal contact via a narrow apical process. Using formaldehyde-fixed material it could be shown that the immunoreactive cells, which displayed the apple-green fluorescence typical of fluorescein, were distinct from the 5-HT-containing enterochromaffin cells which exhibited yellow formaldehyde-induced fluorescence (see Björklund et al., 1972). No neurotensin cells could be demonstrated in the stomach, colon or pancreas, and they were rarely seen in the duodenum. No immunoreactive nerves could be detected in the gut wall.

In the chicken, neurotensin cells were found throughout the intestine. For orientation, the anatomy of the avian digestive tract is illustrated in Figure 8. The immunoreactivity was equally well preserved with formaldehyde or DEPC fixation. Also in the chicken the neurotensin cells were distinct from the enterochromaffin cells. The neurotensin cells were most numerous in the antrum (gizzard-duodenal junction, Fig. 9). Here, they seemed to comprise a mixture of intensely immunoreactive and weakly immunoreactive cells in about equal proportions. Neurotensin cells were also regularly found in the colon, but were rare in the caeca. No immunoreactive cells were found in the proventriculus or pancreas. On the whole, neurotensin cells seemed to be more numerous in the avian gut than in the mammalian gut. As in mammals, there was no evidence of neurotensin nerves in the digestive tract of the chicken.

Neurotensin-immunoreactive cells appeared late in the development of both rat and chicken. In rats, they were first observed immediately before birth, having the same distribution as in adult animals. By four to five days after birth, the immunoreactive cells were about as numerous as in adults. In the chicken, neurotensin cells were first seen in the colon of the 18-day embryo. They appeared in the small intestine and in the gizzard-duodenal junction at about the 20th day of incubation, i.e. 1 to 2 days before hatching. Already a few days after hatching the intestines contained the adult number (per unit area) of neurotensin cells.



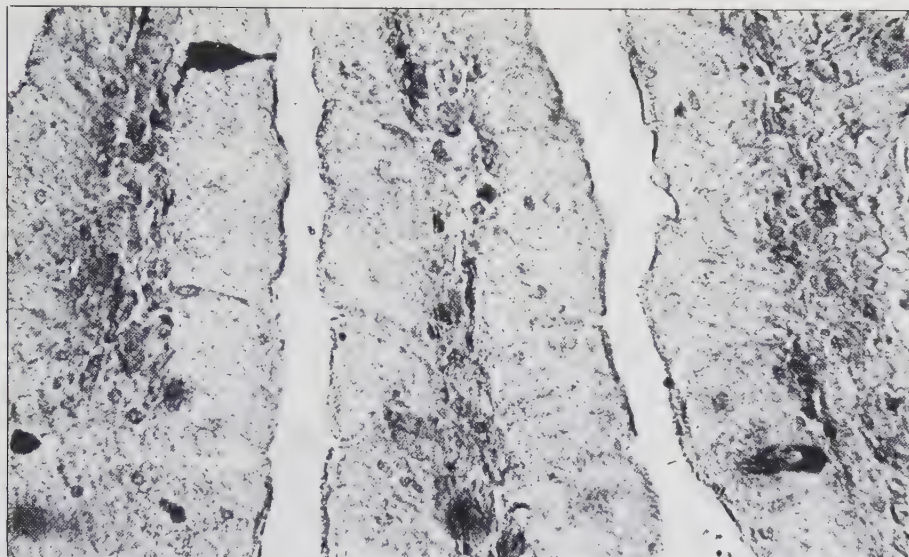


Fig. 7. Guinea pig ileum, formaldehyde fixation. PAP-staining. Two neurotensin-immunoreactive cells, one with an apical process reaching the lumen. $\times 460$

Fig. 1. Rat jejunum, DEPC fixation. Single immunofluorescent neurotensin cell within the epithelium of a villus. $\times 250$

Fig. 2. Rabbit ileum, DEPC fixation. Neurotensin immunofluorescence in cell located in the epithelium of a villus. $\times 315$

Fig. 3. Dog ileum, formaldehyde fixation. Several cells exhibiting neurotensin immunofluorescence in crypt epithelium. $\times 270$

Fig. 4. Dog ileum, DEPC fixation. Neurotensin cells in crypt epithelium. The elongated cell shape indicates that they are of the "open" type, reaching the lumen via a narrow apical process. $\times 400$

Fig. 5. Human ileum, DEPC fixation. Neurotensin cells (*arrows*) in the epithelium of villi. $\times 250$

Fig. 6. Human ileum, formaldehyde fixation. Neurotensin cell at high magnification showing the basal localization of the immunoreactive material. $\times 600$

Table 1. Occurrence and Topographic Distribution of Neurotensin Cells in the Jejunum-Ileum of Some Mammals

Species	Crypts	Villi
Rat	0	+
Rabbit	0	+
Guinea pig	0	+
Cat	+	+
Dog	+	+
Pig	0	+
Man	+	+

0=no cells; +=occasional cell, not present in every section; ++=few cells but present in every section; +++=moderate to high number

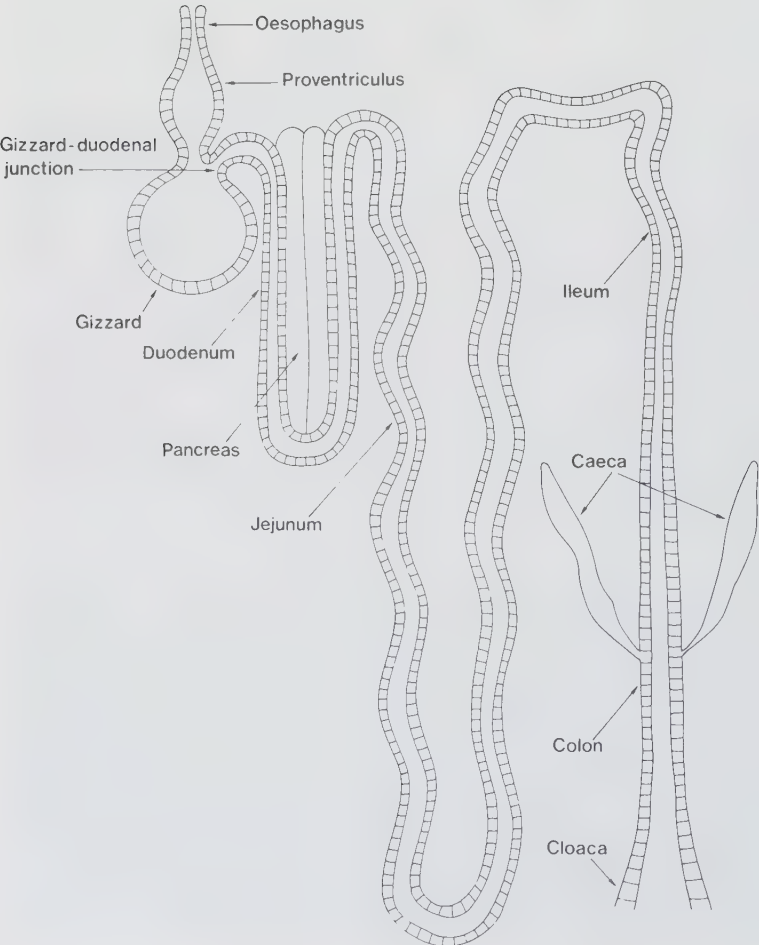


Fig. 8. Schematic drawing of the chicken digestive tract with the various parts indicated by arrows

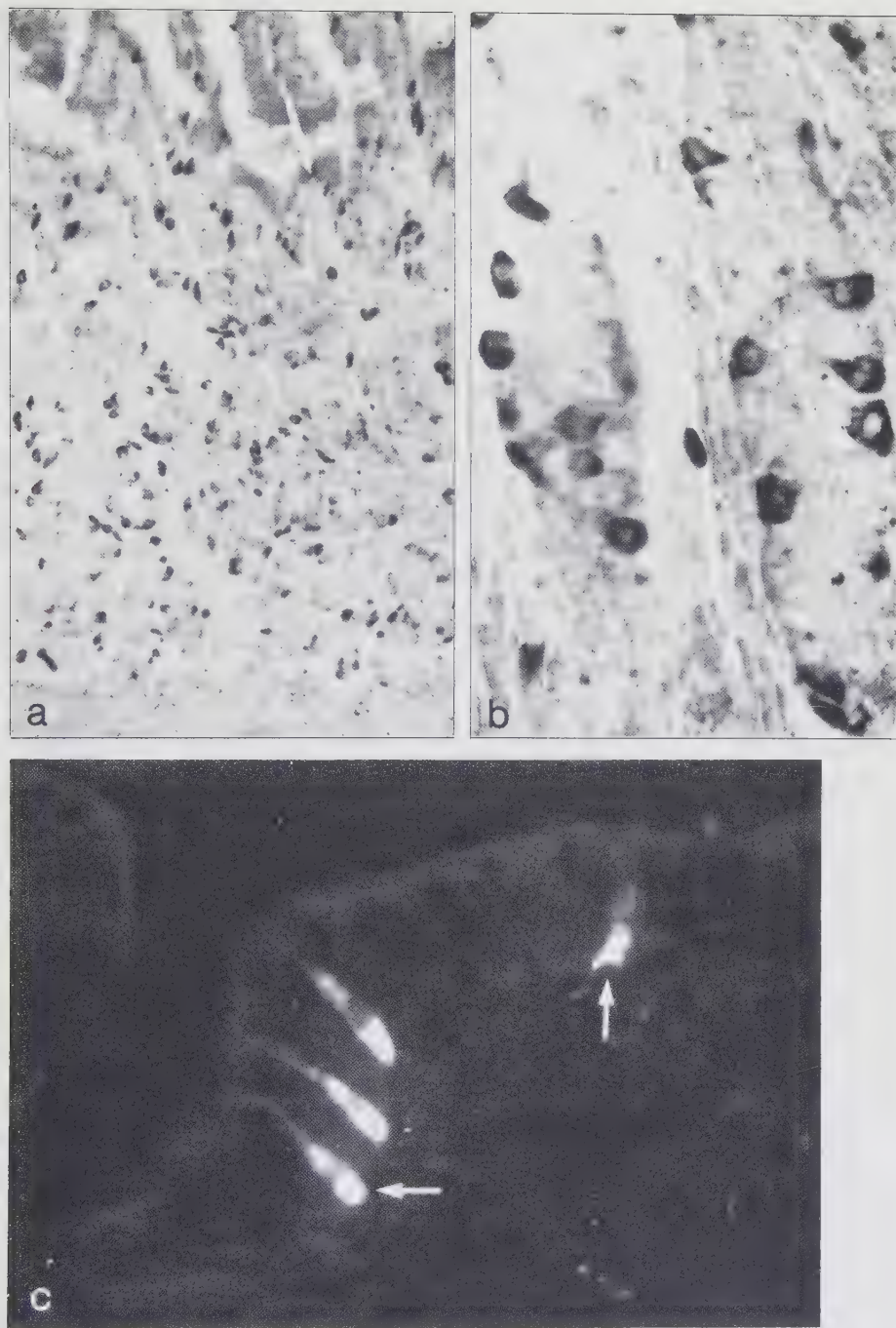


Fig. 9. **a** and **b** Chicken antrum (gizzard-duodenal junction), formaldehyde fixation. Numerous neurotensin cells in the epithelium. PAP-staining. $\times 125$; $\times 700$. **c** Chicken colon, formaldehyde fixation. Immunofluorescent neurotensin cells (apple-green color) together with 5-HT-containing enterochromaffin cells (arrows) displaying formaldehyde-induced fluorescence (yellow color). $\times 625$

Discussion

Cells displaying neurotensin-like immunoreactivity occur in the digestive tract of all species examined. Their distribution in mammals differs from that in the chicken. In mammals, neurotensin-immunoreactive cells are confined to the jejuno-ileum. In the chicken, these cells are found from the gizzard-duodenal junction down to the cloaca; they are particularly numerous in the gizzard-duodenal junction. In both chicken and rat, neurotensin cells cannot be detected until rather late in development.

In view of the potent biological activities of neurotensin and its presence in blood (Carraway and Leeman, 1976b) and in endocrine cells of the small intestines, it is tempting to speculate that neurotensin is involved in the regulation of gastrointestinal functions. Its distribution in the brain as well as in the gut makes neurotensin a member of a rapidly growing list of peptides with such a dual distribution. Other peptides currently on the list are substance P (Pearse and Polak, 1975; Nilsson et al., 1975), somatostatin (Arimura et al., 1975; Hökfelt et al., 1975), VIP (vasoactive intestinal peptide) (Bryant et al., 1976; Larsson et al., 1976a; Larsson et al., 1976b; Said and Rosenberg, 1976) and leucine-enkephalin (Elde et al., 1976).

While this paper was in preparation, Orci et al. (1976) reported their findings of neurotensin-like immunoreactivity in discrete epithelial cells in the dog ileum. The present findings are in full agreement.

Acknowledgements. Grant support from the Swedish Medical Research Council (04X-4499-03) and Riksföreningen mot Cancer (806-03XB), from the National Institutes of Health (AM05381-01A1, AM19428-01, and AM16510-04) and from Pahlsson's Stiftelse and J. and A. Persson's Stiftelse, is gratefully acknowledged.

References

- Andersson, S., Chang, D., Folkers, K., Rosell, S.: Inhibition of gastric acid secretion in dogs by neurotensin. *Life Sci.* **19**, 367-370 (1976)
- Arimura, A., Sato, H., Dupont, A., Nishi, N., Schally, A.V.: Somatostatin: abundance of immunoreactive hormone in rat stomach and pancreas. *Science* **189**, 1007-1009 (1975)
- Björklund, A., Falck, B., Owman, Ch.: Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic monoamines. In: Berson, S.A. (ed.), *Methods of investigative and diagnostic endocrinology*, Vol. 1. J.E. Rall and I.J. Kopin (eds.). The thyroid and biogenic amines, pp. 318-368. Amsterdam: North-Holland Publ. Comp. 1972
- Bryant, M.G., Bloom, S.R., Polak, J.M., Albuquerque, R.H., Modlin, I., Pearse, A.G.E.: Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. *Lancet* **1976 i**, 991-993
- Carraway, R., Demers, L., Leeman, S.E.: Hyperglycemic effect of neurotensin, a hypothalamic peptide. *Endocrinol.* **99**, 1452-1462 (1976)
- Carraway, R., Leeman, S.E.: The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalamus. *J. biol. Chem.* **248**, 6854-6861 (1973)
- Carraway, R., Leeman, S.E.: The amino acid sequence of a hypothalamic peptide, neurotensin. *J. biol. Chem.* **250**, 1907-1911 (1975a)

- Carraway, R., Leeman, S.E.: Structural requirements for the biological activity of neurotensin, a new vasoactive peptide. In: *Peptides: Chemistry, structure and biology* (eds. R. Walter and J. Meienhofer), pp. 679–685. Ann Arbor, Mich.: Ann Arbor Science 1975b
- Carraway, R., Leeman, S.E.: Radioimmunoassay of neurotensin, a hypothalamic peptide. *J. biol. Chem.* **251**, 7035–7044 (1976)
- Carraway, R., Leeman, S.E.: Characterization of radioimmunoassayable neurotensin in the rat; its differential distribution in the central nervous system, small intestine, and stomach. *J. biol. Chem.* **251**, 7045–7052 (1976)
- Elde, R., Hökfelt, T., Johansson, O., Terenius, L.: Immunohistochemical studies using antibodies to leucine-enkephalin: Initial observations on the nervous system of the rat. *Neuroscience* **1**, 349–351 (1976)
- Hökfelt, T., Efendić, S., Hellerström, C., Johansson, O., Luft, R., Arimura, A.: Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special references to the A₁-cells of the pancreatic islets and to the hypothalamus. *Acta endocr. (Kbh.)* **80**, Suppl. 200, 1–41 (1975)
- Kitabgi, P., Carraway, R., Leeman, S.E.: The isolation of tridecapeptide, from bovine intestinal tissue and its partial characterization as neurotensin. *J. biol. Chem.* **251**, 7053–7058 (1976)
- Larsson, L.-I., Edvinsson, L., Fahrenkrug, J., Håkanson, R., Owman, Ch., Schaffalitzky de Muckadell, O., Sundler, F.: Immunohistochemical localization of a vasodilatory polypeptide (VIP) in cerebrovascular nerves. *Brain Res.* **113**, 400–404 (1976a)
- Larsson, L.-I., Fahrenkrug, J., Schaffalitzky de Muckadell, O., Sundler, F., Håkanson, R., Rehfeld, J.F.: Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. *Proc. nat. Acad. Sci. (Wash.)* **73**, 3197–3200 (1976b)
- Larsson, L.-I., Holst, J.J., Håkanson, R., Sundler, F.: Distribution and properties of glucagon immunoreactivity in the digestive tract of various mammals: An immunohistochemical and immunochemical study. *Histochemistry* **44**, 281–290 (1975)
- Nilsson, G., Larsson, L.-I., Håkanson, R., Brodin, E., Pernow, B., Sundler, F.: Localization of substance P-like immunoreactivity in the mouse gut. *Histochem.* **43**, 97–99 (1975)
- Orci, L., Baetens, O., Rufener, C., Brown, M., Vale, W., Guillemin, R.: Evidence for immunoreactive neurotensin in dog intestinal mucosa. *Life Sci.* **19**, 559–562 (1976)
- Pearse, A.G.E., Polak, J.M.: Immunocytochemical localization of substance P in mammalian intestine. *Histochem.* **41**, 373–375 (1975)
- Said, S.I., Rosenberg, R.N.: Vasoactive intestinal polypeptide: Abundant immunoreactivity in neural cell lines and normal nervous tissue. *Science* **192**, 907–908 (1976)
- Sternberger, L.A.: *Immunocytochemistry*. New Jersey: Prentice-Hall Inc. 1974

Accepted December 20, 1976

Ultrastructure of the Gut Neurotensin Cell

F. Sundler¹, J. Alumets¹, R. Håkanson², R. Carraway³, and S.E. Leeman³

Departments of Histology¹ and Pharmacology², University of Lund, Lund, Sweden,
and Department of Physiology and Laboratory of Human Reproduction
and Reproductive Biology³, Harvard Medical School, Boston, Mass., USA

Summary. In mammals, neurotensin cells occur scattered in the epithelium of the jejunum-ileum. In chicken, neurotensin cells are abundant in the region of the gizzard-duodenal junction (antrum) where they occur intermingled with numerous somatostatin and gastrin cells. The neurotensin cells in chicken, dog and man were identified at the electron microscopic level by immunocytochemistry, using the consecutive semithin/ultrathin section technique. They contain numerous electron dense cytoplasmic granules, predominantly in the basal portion of the cell. It was shown that these granules are the storage site for neurotensin. The neurotensin granules are round, highly electron dense and of about the same size in the different species examined (mean diameter 260–290 nm). In dog and man the granules have a tightly applied surrounding membrane while in the chicken a relatively electron lucent zone separates the electron dense core from the granule membrane. The ultrastructure of the neurotensin granules in chicken is somewhat reminiscent of that of the gastrin granules. The mean diameter of the gastrin granules in chicken antrum is 230 nm; for the somatostatin granules the mean diameter is 305 nm.

Introduction

Neurotensin is a tridecapeptide first isolated from the hypothalamus (Carraway and Leeman, 1973), but recently found to occur also in the gut (Carraway and Leeman, 1976a, b; Kitabgi et al., 1976). By immunohistochemistry neurotensin has been localized to a population of endocrine cells in the avian and mammalian gut (Orci et al., 1976; Sundler et al., 1977). In mammals the neurotensin cells are confined to the jejunum and ileum, whereas in the chicken they occur in small and large intestines as well as in the antrum (gizzard-duodenal junction). In the latter location the cells displaying neurotensin immunoreactivity are particularly numerous and occur intermingled with equally numerous gastrin (Larsson et al., 1974a) and somatostatin cells (Alumets et al., 1977). The present report describes the ultrastructure of the neurotensin cell in the gut of chicken, dog and man.

Materials and Methods

Antisera. The neurotensin antiserum (Code No. HC-8) has been characterized in detail elsewhere (Carraway and Leeman, 1976a). It was used in dilution 1:40 or 1:400. The gastrin antiserum (No. 4562) was donated by Dr. J.F. Rehfeld, Department of Biochemistry, Aarhus, Denmark, and used in dilution 1:640. The somatostatin antiserum was generously supplied by Dr. M.P. Dubois, Station de Physiologie de la Reproduction, Nouzilly, France. It was used in dilution 1:40. For details on this antiserum see Dubois (1975). For controls, the antisera were inactivated by the addition of antigen in excess (10 µg neurotensin, 10 µg synthetic human gastrin I or 100 µg synthetic ovine somatostatin per ml diluted antiserum). Fluorescein-labeled and unlabeled anti-rabbit IgG, raised in sheep, were purchased from Statens Bakteriologiska Laboratorium, Stockholm, Sweden, and used in dilution

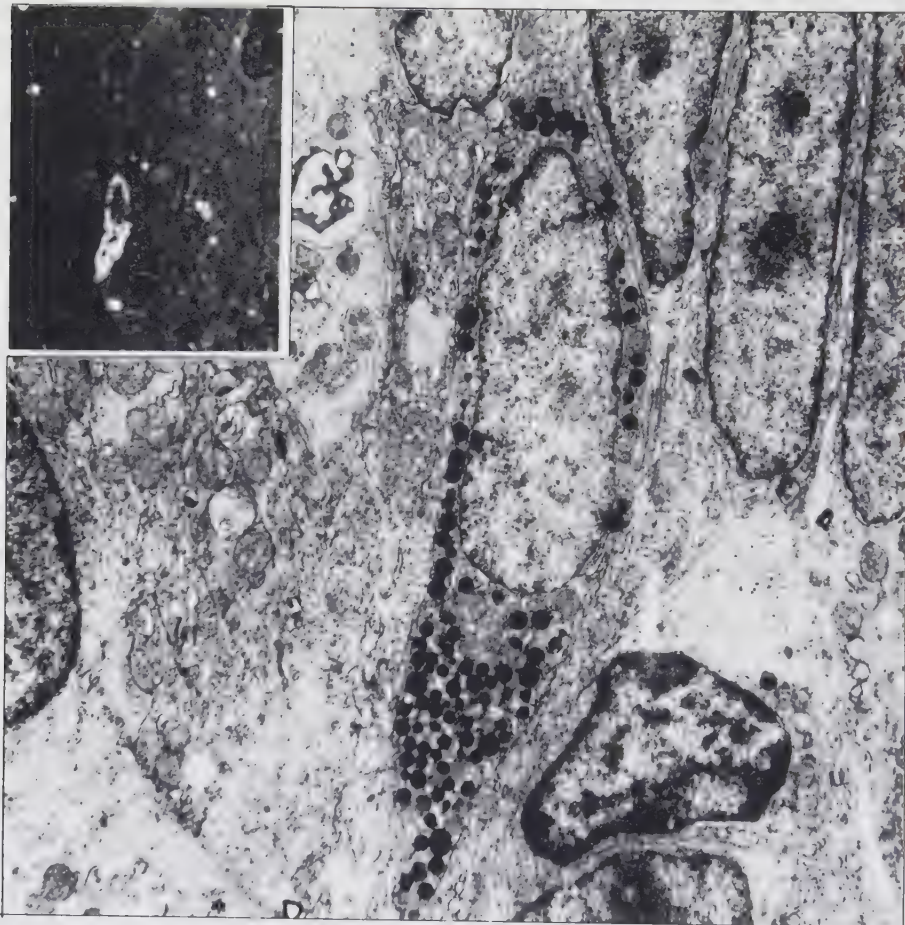


Fig. 1. Immunocytochemical identification of neurotensin cell in dog ileum. Formaldehyde-glutaraldehyde-osmium tetroxide fixation. Epon. Consecutive semithin/ultrathin section technique. Insert: Immunofluorescence of neurotensin cell in semithin section. $\times 600$. The electron micrograph demonstrates fine structural details of the same cell in an adjacent ultrathin section. Note characteristic electron dense cytoplasmic granules accumulated basally in the cell. $\times 7500$

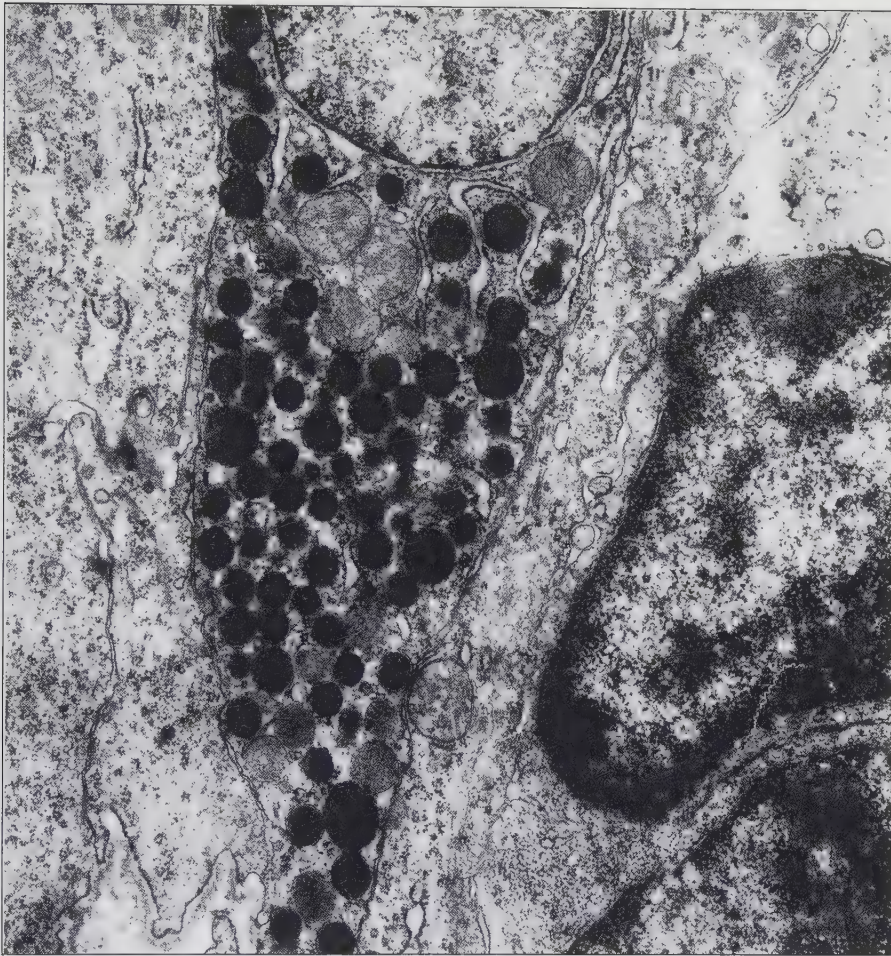


Fig. 2. Neurotensin cell in dog ileum. Same cell as in Figure 1. Formaldehyde-glutaraldehyde-osmium tetroxide fixation. Ultrastructure of cytoplasmic granules ($\times 19,000$)

1:20. Peroxidase-antiperoxidase complex (PAP) was obtained from Cappel Laboratories, Downington, Pennsylvania, USA. It was used in dilution 1:80.

Tissue Processing. The following species were examined: chicken, dog and man. Anesthetized chickens (6 to 8 weeks old) were perfused via the heart for 5 min with a fixation solution containing 3% formaldehyde and 1% glutaraldehyde in 0.075 M or 0.1 M phosphate buffer, pH 7.2. Small specimens from the antrum (gizzard-duodenal junction) were dissected out and immersed overnight (16–18 h) in the same fixative. Specimens from dog ileum and human jejunum and ileum (resected for intestinal carcinoma; courtesy of Drs. S. Ingemansson and G. Liedberg, Department of Surgery, University Hospital, Lund, Sweden) were fixed by immersion overnight in the same type of fixative as described above. All specimens were postfixed for 2 h in 1% osmium tetroxide, dehydrated in graded ethanol solutions, contrasted en bloc with 1% phosphotungstic acid and 0.5% uranyl acetate in ethanol and embedded in Epon.

Immunocytochemistry. Semithin ($<0.5\mu$) sections were mounted on glass slides. The plastic was removed with sodium methoxide according to Mayor et al. (1961). Osmium deposits were removed by 1% periodic acid (see Beauvillain et al., 1975). The sections were then processed for the immunohistochemical demonstration of neurotensin (Sundler et al., 1977), gastrin (Larsson et al., 1974b) or somatostatin (Dubois, 1975; Alumets et al., 1977). After exposure to antiserum for 30 min at room temperature, the sections were rinsed in phosphate buffered saline, pH 7.2. The site of antigen-antibody reaction was revealed by incubation with fluoresceinated anti-rabbit IgG. Sections processed for immunofluorescence were mounted in buffered glycerine and examined in a Leitz Orthoplan fluorescence microscope equipped with a Ploem epi-illumination system (filter setting No. 3; excitation peak at 490 nm). Ultrathin sections were placed on grids and contrasted with lead citrate and uranyl acetate and examined in a Philips EM 300 electron microscope. Immunoreactive cells present in semithin sections were identified in adjacent ultrathin sections (consecutive semithin/ultrathin section technique) (Beauvillain et al., 1975; Sundler et al., 1976). The granule size was established in endocrine cells in chicken antrum and dog ileum by measuring the diameter of all granule profiles in at least 8 identified cells (unless otherwise stated) of each type from each species. The photomicrographs were reproduced at a magnification of $15,000\times$.

In one study ultra-thin plastic sections were etched in 10% hydrogen peroxide for 20 min and incubated with anti-neurotensin serum in dilution 1:400. The site of antigen-antibody reaction was revealed by PAP staining according to the technique of Sternberger (1974). For details see Dacheux and Dubois (1976).

Results

In the small intestines of man and dog the cells displaying neurotensin immunoreactivity contained numerous homogenous, uniformly electron dense cytoplasmic granules (Figs. 1, 2). They usually predominated in the basal part of the cells and were round or slightly angular. The surrounding membrane was closely applied to the dense core. The mean diameter of neurotensin cell granules in dog ileum was 260 ± 64 (S.D.) ($n = 890$) (Fig. 3). From examination of three identified neurotensin cells in human intestines it appears that the granular size in man ($290 \text{ nm} \pm 76$, $n = 107$) is slightly larger than in the dog. The difference is not statistically significant, however.

In the chicken antrum endocrine cells were abundant. The neurotensin cells seemed to comprise two populations, one strongly immunoreactive and another only moderately immunoreactive (Sundler et al., 1977). Besides the neurotensin

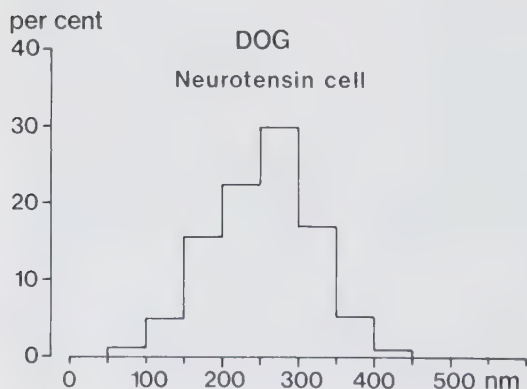


Fig. 3. Size distribution histogram of the granules of neurotensin cells in dog intestines. For details see text

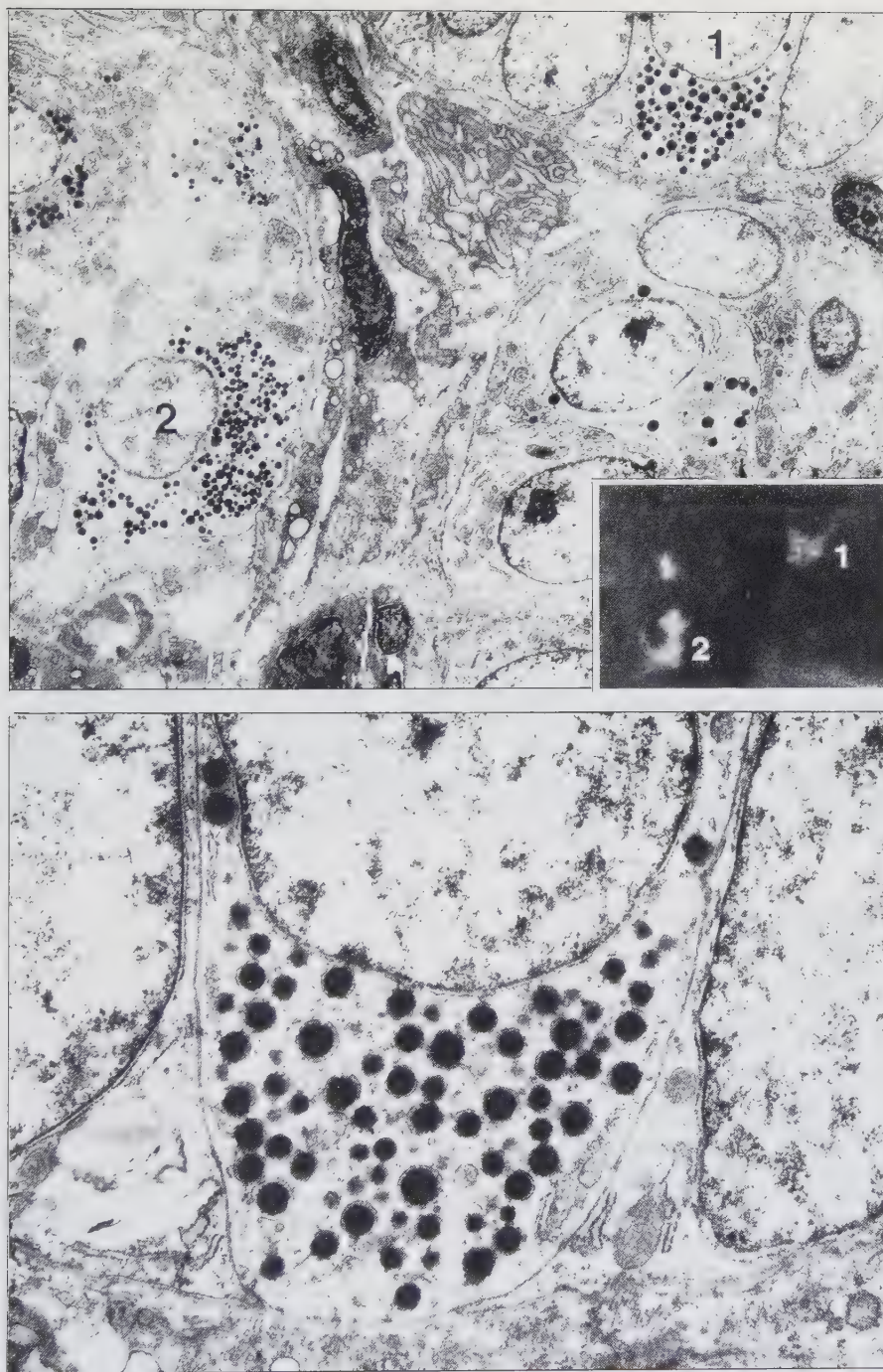


Fig. 4. Neurotensin cells in chicken antrum identified by the consecutive semithin/ultrathin section technique as in Figure 1. Formaldehyde-glutaraldehyde osmium tetroxide fixation. Insert: Immunofluorescence of neurotensin cells (labelled 1, 2). $\times 600$. The low magnification electron micrograph (above) shows the same cells (1 and 2) in an adjacent ultrathin section. Granules are found basally in the two cells. $\times 4300$. High magnification electron micrograph (below) of cell no 1 demonstrates fine structural details. $\times 16,000$

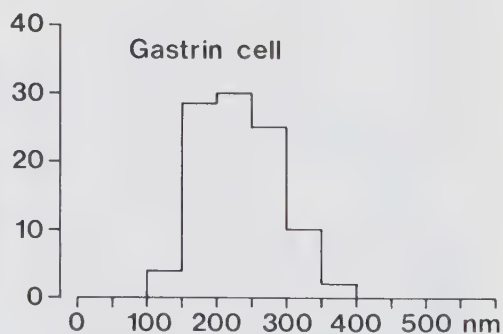
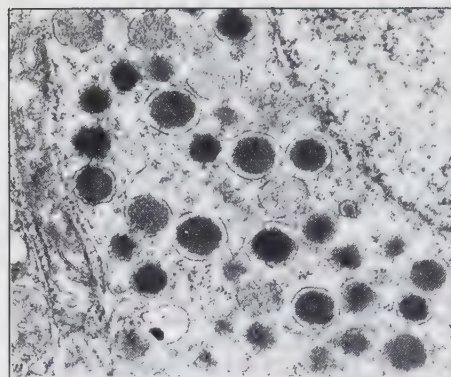
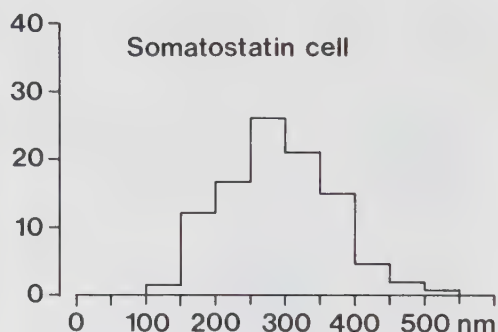
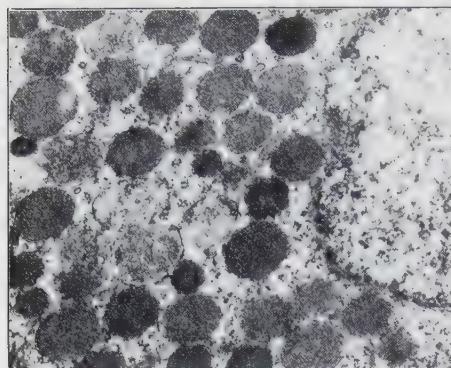
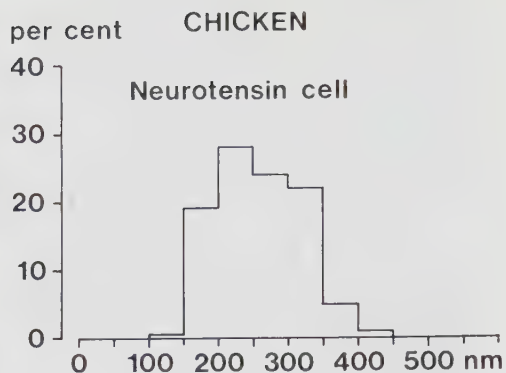
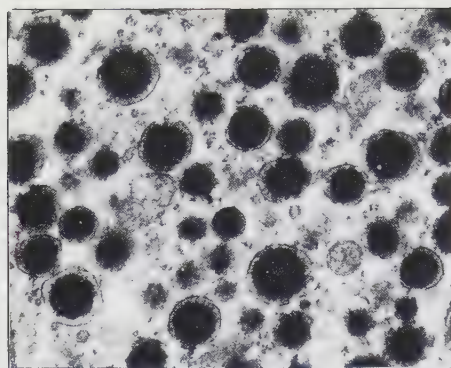


Fig. 5. Chicken antrum. Formaldehyde-glutaraldehyde-osmium tetroxide fixation. Ultrastructure of cytoplasmic granules of neurotensin cell, somatostatin cell and gastrin cell (all identified by the consecutive semithin/ultrathin section technique) ($\times 33,000$) together with granular size distribution histograms. For details see text. Note presence of electron dense as well as electron lucent granules in the gastrin cell. Note also the electron lucent zone between the dense core and the surrounding membrane of most gastrin granules. The corresponding zone in the neurotensin granules is more electron dense

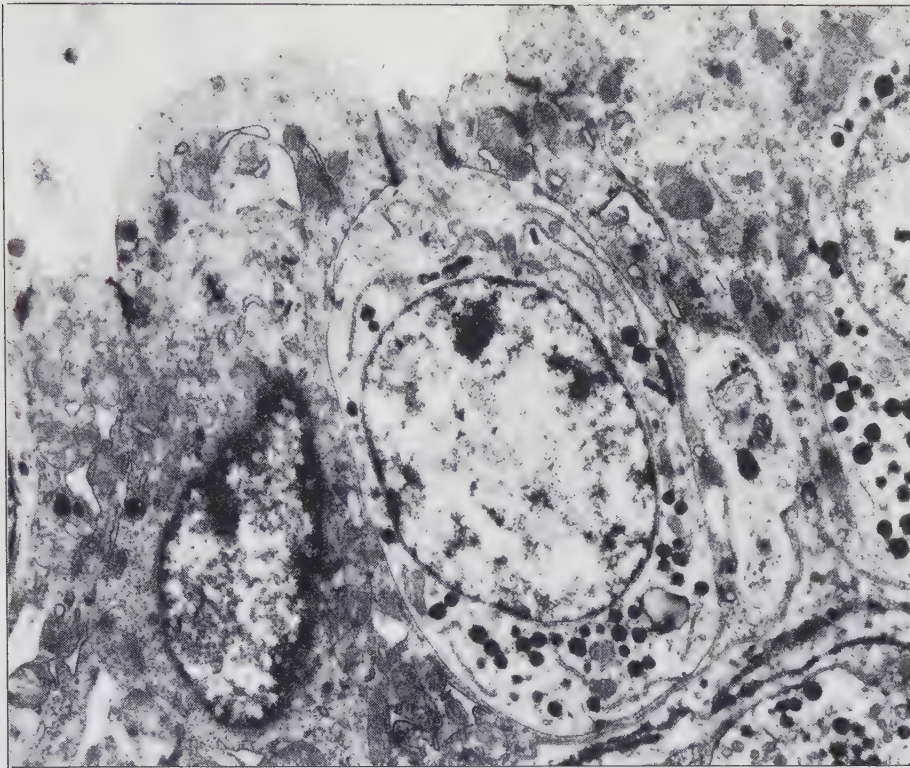


Fig. 6. Neurotensin cell in chicken antrum, identified by the consecutive semithin/ultrathin section technique. The narrow apical portion of the cell, reaching the lumen, is endowed with microvilli. ($\times 9000$)

cells, somatostatin cells and gastrin cells occurred in large numbers. The granules of the strongly immunoreactive neurotensin cell differed from those of its mammalian counterpart in that they exhibited a relatively electron lucent zone between the electron dense core and the surrounding membrane (Figs. 4 and 5). The granular mean diameter was $260 \text{ nm} \pm 59 \text{ (S.D.)}$ ($n = 652$) (Fig. 5). The granules of the somatostatin cells were round, homogenous and with a varying, often low, electron density. The membrane was closely applied to the dense core which displayed a granulated texture. The mean diameter of the somatostatin granules was $305 \pm 78 \text{ (S.D.)}$ ($n = 592$) (Fig. 5). The granules of the gastrin cell were somewhat smaller ($230 \pm 54 \text{ (S.D.)}$; $n = 548$) than those of the neurotensin and somatostatin cells (Fig. 5). The gastrin granules were round, most often highly electron dense, and usually displayed an electron lucent zone between the membrane and the dense core.

In the chicken antrum, neurotensin cells were often seen to reach the lumen via a short process, usually furnished with microvilli (Fig. 6). Due to the thickness of the epithelium in the mammals it was usually not possible to trace in a single section the apical process of a neurotensin cell as it extends towards the lumen.

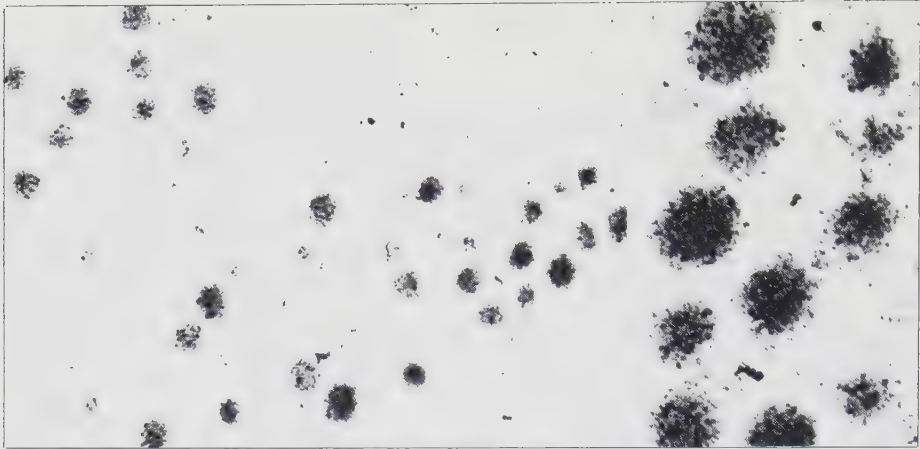


Fig. 7. Neurotensin cell in chicken antrum. PAP staining of section from formaldehyde-glutaraldehyde-osmium tetroxide fixed specimen. The section was etched with hydrogen peroxide in order to remove plastic and osmium precipitates. The anti-neurotensin serum was used in dilution 1:400. The cytoplasmic granules are heavily loaded with electron dense material indicating the presence of neurotensin (left $\times 16,000$; right $\times 33,000$)

However, by examining serial sections we could show that also the neurotensin cells of the mammalian gut reach the lumen via a narrow neck, its apical surface being endowed with microvilli.

After PAP-staining the cytoplasmic granules of the chicken neurotensin cells were heavily loaded with electron dense precipitates (Fig. 7).

Discussion

Neurotensin in the gut occurs exclusively in endocrine cells which are rich in characteristic cytoplasmic granules. They represent the intracellular storage site for the peptide. In mammals, the granules are highly electron dense and surrounded by a tightly applied membrane; their mean diameter is 260–290 nm. In previous reports on the ultrastructure of endocrine cells in the mammalian gut the neurotensin cell has probably been grouped together with other cells with fairly large, electron dense granules under the name of L cells (Vassallo et al., 1969; Kubes et al., 1974; Solcia et al., 1975). The possible heterogeneity of the intestinal L cells was anticipated already in 1969 by Vassallo et al. In mammals, neurotensin cells are found only in the jejunum-ileum. The L cell, which occurs also in other gut segments than jejunum and ileum, has been tentatively associated with the production and secretion of enteroglucagon and has therefore been labelled EG cell (Polak et al., 1971). The intestinal cells, which are reactive to glucagon antisera (i.e. EG cells), are clearly distinct from the neurotensin cells as demonstrated by serial sectioning of plastic embedded material (Sundler, Alumets and Håkanson,

unpublished observation). The similarity between the granules of the neurotensin cell and those of the L cell (Solcia et al., 1975) emphasizes the difficulties involved in classifying gut endocrine cells by their ultrastructure alone.

Neurotensin cells are abundant in the chicken antrum where they occur intermingled with somatostatin and gastrin cells which also are numerous in this region (Alumets et al., 1977; Larsson et al., 1974a). The neurotensin granules in chicken are highly electron dense and of about the same size as those in mammals. However, the ultrastructure of the granules is different: a distinct zone of moderate to low electron density invariably separates the electron dense core from the surrounding membrane. The neurotensin granules in chicken are somewhat similar to the gastrin granules in size, ultrastructure, and the way the membrane is applied to the dense core. They are markedly different, however, from the somatostatin granules. It is noteworthy that the ultrastructure of the chicken gastrin granules in turn differs from that of the mammalian gastrin granules (Solcia et al., 1975).

Acknowledgements. Grant support from the Swedish Medical Research Council (04X-4499) and Pahlsson's Foundation.

References

- Alumets, J., Sundler, F., Håkanson, R.: Distribution, ontogeny and ultrastructure of somatostatin immunoreactive cells in pancreas and gut. *Cell Tiss. Res.*, in press (1977)
- Beauvillain, J.C., Tramu, G., Dubois, M.P.: Characterization by different techniques of adreno-corticotropin and gonadotropin producing cells in Lerot pituitary (*Eliomys quercinus*). *Cell Tiss. Res.* **158**, 301–317 (1975)
- Carraway, R., Leeman, S.E.: The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J. biol. Chem.* **248**, 6854–6861 (1973)
- Carraway, R., Leeman, S.E.: Radioimmunoassay of neurotensin, a hypothalamic peptide. *J. biol. Chem.* **251**, 7035–7044 (1976a)
- Carraway, R., Leeman, S.E.: Characterization of radioimmunoassayable neurotensin in the rat; its differential distribution in the central nervous system, small intestine and stomach. *J. biol. Chem.* **251**, 7045–7052 (1976b)
- Dacheux, F., Dubois, M.P.: Ultrastructural localization of prolactin, growth hormone and luteinizing hormone by immunocytochemical techniques in the bovine pituitary. *Cell Tiss. Res.* **174**, 245–260 (1976)
- Dubois, M.P.: Presence of immunoreactive somatostatin in discrete cells of the endocrine pancreas. *Proc. Natl. Acad. Sci. USA* **72**, 1340–1343 (1975)
- Kitabgi, P., Carraway, R., Leeman, S.E.: The isolation of a tridecapeptide from bovine intestinal tissue and its partial characterization as neurotensin. *J. biol. Chem.* **251**, 7053–7058 (1976)
- Kubes, L., Jirasek, K., Lomsky, K.: Endocrine cells of the dog gastrointestinal mucosa. *Cytologia (Tokyo)* **39**, 179–194 (1974)
- Larsson, L.I., Sundler, F., Håkanson, R., Grimelius, L., Rehfeld, J.F., Stadil, F.: Histochemical properties of the antral gastrin cell. *J. Histochem. Cytochem.* **22**, 419–427 (1974b)
- Larsson, L.I., Sundler, F., Håkanson, R., Rehfeld, J.F., Stadil, F.: Distribution and properties of gastrin cells in the gastrointestinal tract of chicken. *Cell Tiss. Res.* **154**, 409–421 (1974a)
- Mayor, H.D., Hampton, J.C., Rosario, B.: A simple method for removing the resin from epoxy-embedded tissue. *J. biophys. biochem. Cytol.* **9**, 909–910 (1961)
- Orci, L., Baetens, O., Rufener, C., Brown, M., Vale, W., Guillemin, R.: Evidence for immunoreactive neurotensin in dog intestinal mucosa. *Life Sci.* **19**, 559–562 (1976)

- Polak, J.M., Bloom, S., Coulling, I., Pearse, A.G.E.: Immunofluorescent localization of entero-glucagon cells in the gastrointestinal tract of the dog. *Gut* **12**, 311–318 (1971)
- Solcia, E., Capella, C., Vassallo, G., Buffa, R.: Endocrine cells of the gastric mucosa. *Int. Rev. Cytol.* **42**, 223–286 (1975)
- Sternberger, L.A.: *Immunocytochemistry*. Inc., Englewood Cliffs, NJ.: Prentice-Hall 1974
- Sundler, F., Alumets, J., Holst, J., Larsson, L.-I., Håkanson, R.: Ultrastructural identification of cells storing pancreatic-type glucagon in dog stomach. *Histochemistry* **50**, 33–37 (1976)
- Sundler, F., Håkanson, R., Hammer, R.A., Alumets, J., Carraway, R., Leeman, S.E., Zimmerman, E.: Immunohistochemical localization of neurotensin in endocrine cells of the gut. *Cell Tiss. Res.* **178**, 313–321 (1977)
- Vassallo, G., Solcia, E., Capella, C.: Light and electron microscopic identification of several types of endocrine cells in the gastrointestinal mucosa of the cat. *Z. Zellforsch.* **98**, 333–356 (1969)

Received March 21, 1977

Distribution, Ontogeny and Ultrastructure of Somatostatin Immunoreactive Cells in the Pancreas and Gut

J. Alumets, F. Sundler and R. Håkanson

Departments of Histology and Pharmacology, University of Lund, Lund, Sweden

Summary. Somatostatin cells are numerous in the pancreas and digestive tract of mammals as well as birds. In the pancreas of chicken, cat and dog they occur in both the exocrine parenchyma and in the islets. In the rat and rabbit, somatostatin cells have a peripheral location in the islets, whereas in the cat, dog and man the cells are usually more randomly distributed. In the stomach of rabbits and pigs, somatostatin cells are more numerous in the oxyntic gland area than in the pyloric gland area, whereas the reverse is true for the cat, dog and man. In the cat, pig and man, somatostatin cells are fairly numerous in the duodenum, whereas in the rat, rabbit and dog they are few in this location. In the remainder of the intestines somatostatin cells are few but regularly observed. Somatostatin cells are numerous in the human fetal pancreas and gut. In the fetal rat, somatostatin cells first appear in the pancreas and duodenum (at about the 16–17th day of gestation) and subsequently in the remainder of the intestine. Somatostatin cells do not appear in the gastric mucosa until after birth. Three weeks after birth, somatostatin cells show the adult frequency of occurrence and pattern of distribution. In the chicken, somatostatin cells are numerous in the proventriculus, absent from the gizzard, abundant in the gizzard-duodenal junction (antrum), infrequent in the duodenum and virtually absent from the remainder of the intestines. No immunoreactive cells can be observed in the thyroid of any species nor in the ultimobranchial gland of the chicken. In the chick embryo, somatostatin cells are first detected in the pancreas and proventriculus (at about the 12th day of incubation). They appear in the remainder of the gut much later, in the duodenum at the 16th day, in the antrum at about the 19th day and still later in the lower small intestine. The ultrastructure of the somatostatin cells was studied in the chicken, rat, cat and man; the cells were identified by the consecutive semithin/ultrathin section technique. The somatostatin cells display the properties of the D cell. There was no difference in granule ultrastructure between somatostatin cells in the gut and

Send offprint requests to: Dr. Frank Sundler, Histologiska institutionen, Biskopsgatan 5, S-223 62 Lund/Sweden

the pancreas. The granules, which are the storage site of the peptide, are round, supplied with a tightly fitting membrane and have a moderately electron-dense, fine-granulated core. The mean diameter of the somatostatin granules is smallest in rat (155–170 nm) and largest in the chicken (270–290 nm).

Key words: Somatostatin cells – Pancreas – Gut – Immunocytochemistry – Comparative study.

Introduction

Somatostatin is a tetradecapeptide which was isolated from the hypothalamus and found to block the secretion of growth hormone (Brazeau et al., 1973). Somatostatin-like immunoreactivity was subsequently shown to occur not only in the brain but also in the periphery, notably in endocrine cells of the pancreas and the digestive tract (Luft et al., 1974; Arimura et al., 1975; Dubois, 1975; Dubois et al., 1975; Hökfelt et al., 1975a; Pelletier et al., 1975; Polak et al., 1975; Rufener et al., 1975a, b; Weir et al., 1976). On the basis of ultrastructural studies the cells have been claimed to be identical with the D cells (Goldsmith et al., 1975; Orci et al., 1975; Rufener et al., 1975c; Leclerc et al., 1976). For a recent review see Orci et al. (1976). There have been reports of somatostatin-like immunoreactivity also in a population of thyroid parafollicular cells (Hökfelt et al., 1975a; Parsons et al., 1976) and in intramural nerves of the gut (Hökfelt et al., 1975a, b). The present report summarizes our findings on the distribution, ontogeny and ultrastructural properties of somatostatin cells in peripheral tissues of several species.

Materials and Methods

Antisera. Antiserum against somatostatin was generously donated by Dr. M.P. Dubois, Institut National de la Recherche Agronomique, Nouzilly, France. The antiserum has previously been characterized (Dubois, 1975). Antiserum against synthetic human gastrin I (No. 2609) was kindly supplied by Professor J.F. Rehfeld, Department of Medical Biochemistry, Aarhus University, Aarhus, Denmark. For details on this antiserum see Rehfeld et al. (1972). Fluorescein isothiocyanate-labeled and unlabeled sheep anti-rabbit IgG were obtained from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. The peroxidase-antiperoxidase complex (PAP) was purchased from Cappel Laboratories, Downington, Pennsylvania, USA.

Tissue Material. Five adult Sprague-Dawley rats, three white rabbits, three cats and three mongrel dogs were killed by exsanguination under diethyl ether anesthesia (rats), injection of air (rabbits), or by an overdose of nembutal (cats and dogs). Material from pigs was obtained from a local abattoir. Human material was collected at surgery for gastric ulcer or gastrointestinal carcinoma (courtesy of Drs. S. Ingemansson and G. Liedberg, Department of Surgery, University Hospital, Lund, Sweden). Specimens from human fetuses (15–23 weeks gestational age) were obtained at legal abortions (courtesy of Professor S. Kullander, Department of Gynecology, University Hospital, Malmö, Sweden). Specimens were collected from fetal rats from the 15th day of gestation up to parturition and from newborn and young rats 1–25 days old. The material also included chicken embryos (16–22 days of incubation), newly hatched chickens and chickens 6–10 weeks old. Pancreatic specimens were taken from the splenic and duodenal portion of mammals and from the splenic, dorsal and ventral lobes of chickens. Specimens were taken from various parts of the gastrointestinal tract of all species; from the thyroid of the rat, cat and dog; and the thyroid and ultimobranchial glands from the chicken. All specimens were frozen at the

temperature of liquid nitrogen in a propane-propylene mixture and freeze-dried. They were then exposed to formaldehyde (Björklund et al., 1972) or diethylpyrocarbonate (DEPC) (Pearse and Polak, 1975) vapor and embedded in paraffin in vacuo.

Immunofluorescence and Immunoperoxidase Staining. Sections were cut at 5 μ m, deparaffinized in xylene and carried down to water through graded ethanol solutions. They were then exposed to the somatostatin antiserum in dilution 1:40 for 3 h at room temperature (immunofluorescence) or in dilution 1:640 for 24 h at +4°C (PAP staining). In one series, anti-gastrin serum was applied in dilution 1:20 for 3 h. The site of antigen-antibody reaction was revealed either by fluorescein-labeled anti-rabbit IgG diluted 1:20 (30 min incubation), or by unlabeled anti-rabbit IgG applied at 1:100 dilution for 30 min, followed by incubation for 1 h with PAP (diluted 1:80) and 5 minute exposure to 3,3'-diaminobenzidine, 50 mg/100 ml (for details see Sternberger, 1974). All solutions used contained 0.25% human serum albumin and 0.25% Triton X-100. The sections were rinsed and mounted in phosphate buffered glycerine (immunofluorescence) or dehydrated and mounted in Permount (immunoperoxidase) and examined with a fluorescence or light microscope. Controls included sections exposed to antigen-inactivated antiserum (100 μ g synthetic ovine somatostatin or 10 μ g synthetic human heptadecapeptide gastrin per ml diluted antiserum). In one experiment other peptides were added to the somatostatin antiserum in order to test for signs of inactivation. These peptides included porcine CCK, glucagon, insulin, ACTH, bovine pancreatic polypeptide, synthetic human gastrin I, and substance P (in a concentration of 10–100 μ g peptide per ml diluted antiserum).

Cell Counting. The oxyntic mucosa and the antral mucosa of newborn, young and adult rats were analyzed. The number of cells displaying somatostatin immunoreactivity (PAP staining) was assessed by examining sections cut transversally to the mucosal surface at a magnification of 125 X (objective 10 X, eyepiece 12.5X, visual field diameter 1.4 mm). Cells in five randomly selected visual fields (entire thickness of mucosa visible) from each section were counted. At least four sections from each animal were examined. Cell counts are expressed as number of cells per visual field.

Electron Microscopy and Immunocytochemistry. Material was collected for electron microscopy from the chicken, rat, cat and man. Anesthetized chickens and rats were perfused for 5 min via the heart with a fixation solution containing 3% formaldehyde and 1% glutaraldehyde in 0.075 M phosphate buffer, pH 7.2. Small specimens from the pancreas and from gastric and duodenal mucosa were dissected out and immersed overnight (16–18 h) in the same fixative. Specimens from the cat and man were fixed by immersion overnight in the same fixative as described above. All specimens were postfixed for 2 h in 1% osmium tetroxide, dehydrated in graded ethanol solutions, contrasted *en bloc* with 1% phosphotungstic acid and 0.5% uranyl acetate in ethanol and embedded in Epon. Identification of immunoreactive cells at the ultrastructural level was made by the consecutive semithin/ultrathin section technique. Semithin (< 0.5 μ m) sections were mounted on glass slides. The resin was removed with sodium methoxide according to Mayor et al. (1961). Osmium deposits were removed with 1% periodic acid (see Beauvillain et al., 1975). The sections were then processed for the immunohistochemical demonstration of somatostatin (see above). Adjacent ultrathin sections were placed on grids and contrasted with lead citrate and uranyl acetate and examined in a Philips EM 300 electron microscope. Immunoreactive cells in the semithin section were identified in the adjacent ultrathin sections (consecutive semithin/ultrathin section technique) (cf. Beauvillain et al., 1975). In one experiment somatostatin immunoreactivity was demonstrated by PAP staining of ultrathin sections (for details, see Beauvillain et al., 1975).

The granule size was established by measuring the diameter of all cytoplasmic granule profiles in at least eight identified cells from each location (pancreas and gut) in each species. The photomicrographs used for this purpose were reproduced at a magnification of 15,000 X.

Results

Distribution of Somatostatin-Immunoreactive Cells. Somatostatin-like immunoreactivity could be demonstrated in endocrine cells in the pancreas and digestive tract of all species examined. Both DEPC and formaldehyde vapor fixation

Table 1. Regional distribution and relative frequency of somatostatin cells in the digestive tract

Species	Fundus	Antrum	Duodenum	Jejunum-Ileum		Colon
Rat	++	++	+		+	+
Rabbit	+++	++	+		+	+
Cat	++	+++	++		+	+
Dog	++	+++	+		+	+
Pig	++	++	++		+	+
Man	+	++	++		+	+
	Proventri- culus	Gizzard	Antrum	Duo- denum	Jejunum Ileum	Colon
Chicken	+++	—	++++	+	—	—

++++ = Very numerous; +++ = numerous; ++ = moderately numerous; + = few, but regularly observed; - = absent

preserved the immunoreactivity. With DEPC the reaction intensity was better than with formaldehyde; on the other hand diffusion of the immunoreactive material with DEPC was evident. Addition of somatostatin to the antiserum blocked the immunoreaction, whereas the other peptides tested did not. In all mammals somatostatin cells were numerous in the gastric mucosa; they seemed to predominate in the pyloric gland area of the cat, dog and man and in the oxyntic gland area of the rabbit and pig. In the rat, the number of somatostatin cells per visual field was roughly the same in the oxyntic and in the pyloric mucosa. In cat, pig and man somatostatin cells were fairly numerous in the duodenum, whereas in the rat, rabbit and dog somatostatin cells were few in this location. In the remainder of the intestines of all the mammals somatostatin cells were observed regularly but in small numbers. The regional distribution of the somatostatin-immunoreactive cells in the gut of the various species is described in Table 1. The topographical distribution of the somatostatin cells in the antral mucosa was similar to that of other endocrine cells in this region. Thus, in the rat and rabbit the somatostatin cells predominated in the basal third of the mucosa with scattered cells occurring higher up (Fig. 1a), while in the antrum of the cat, dog and man the cells occurred in a zone located between the lower third and the upper two thirds of the mucosa (Fig. 1b). In the oxyntic mucosa and in the intestines the somatostatin cells generally had a more random distribution (Fig. 2). The somatostatin cells of the intestines of all species appeared to possess an apical process in contact with the lumen (Fig. 2a). This did not seem to be the case with the somatostatin cells in oxyntic mucosa; in the pyloric mucosa only some immunoreactive cells possessed an apical process. Somatostatin cells were fairly numerous in the pancreatic islets of all mammals tested. They were more numerous in the lienal than in the duodenal portion of the pancreas. Usually they had a peripheral location in the islets (Fig. 3a), although in islets of the cat, dog and man they sometimes displayed a fairly random distribution. Somatostatin cells were regularly seen in the ductal epithelium of the human pancreas (Fig. 3b). In the cat and dog, somatostatin cells were also fairly numerous in the exocrine parenchyma, notably in the duodenal portion (Table 2). No immunoreactive nerves

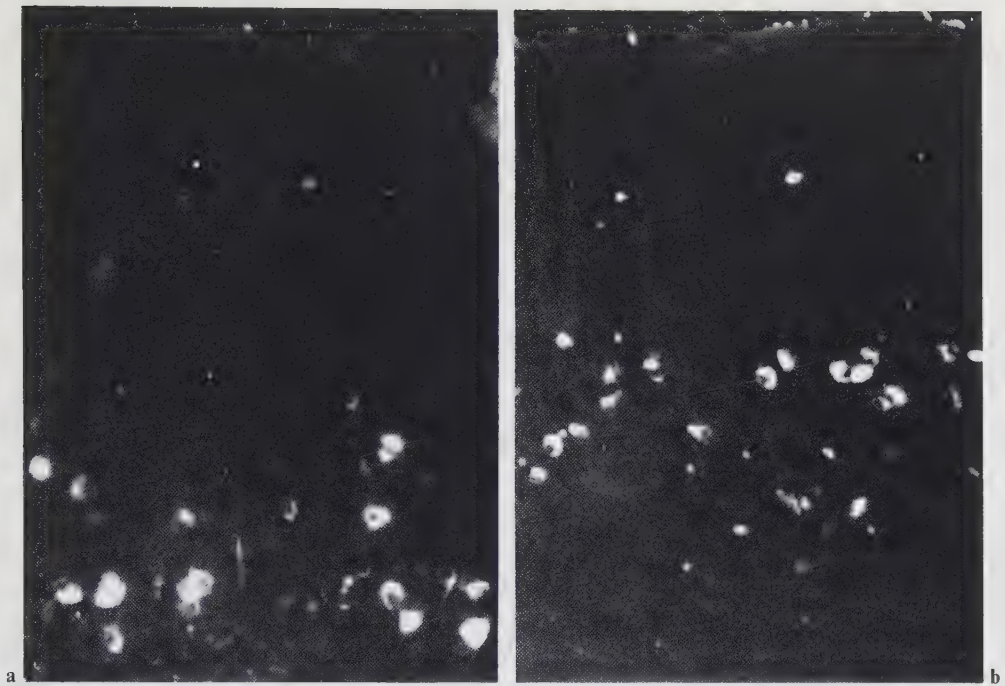


Fig. 1 a and b. Somatostatin immunohistochemistry. **a** Rat antrum, formaldehyde fixation. Green-fluorescent immunoreactive cells together with yellow-fluorescent 5-HT-containing enterochromaffin cells (in roughly equal proportions) in the basal third of the mucosa. $\times 350$. **b** Cat antrum, formaldehyde fixation. Green-fluorescent somatostatin cells and yellow-fluorescent enterochromaffin cells in a zone within the middle third of the mucosa. $\times 250$

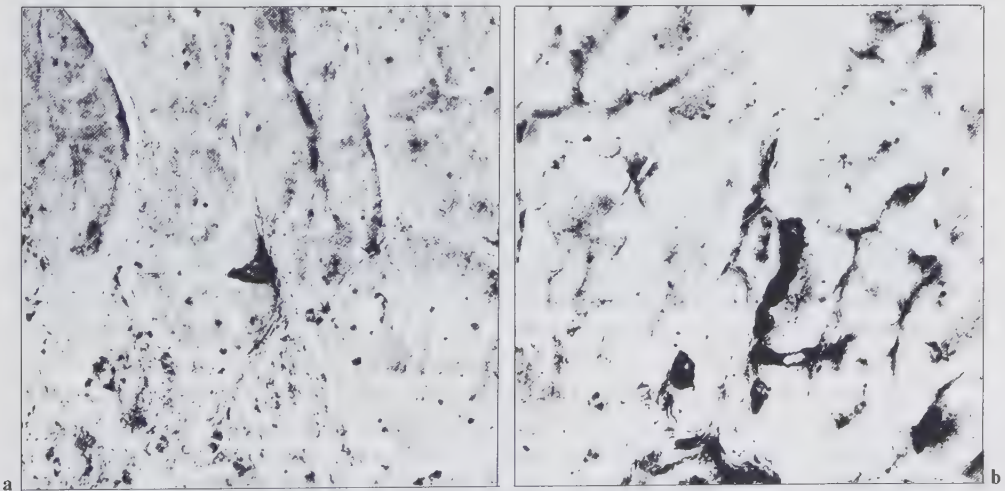


Fig. 2 a and b. Somatostatin immunohistochemistry. **a** Cat jejunum, formaldehyde fixation. Immunoreactive cell demonstrated by PAP staining. Note the elongated apical process which appears to reach the surface of the epithelium. $\times 400$. **b** Rabbit stomach, oxyntic gland area, formaldehyde fixation. Several somatostatin cells lining the base of an oxyntic gland (PAP staining). $\times 350$

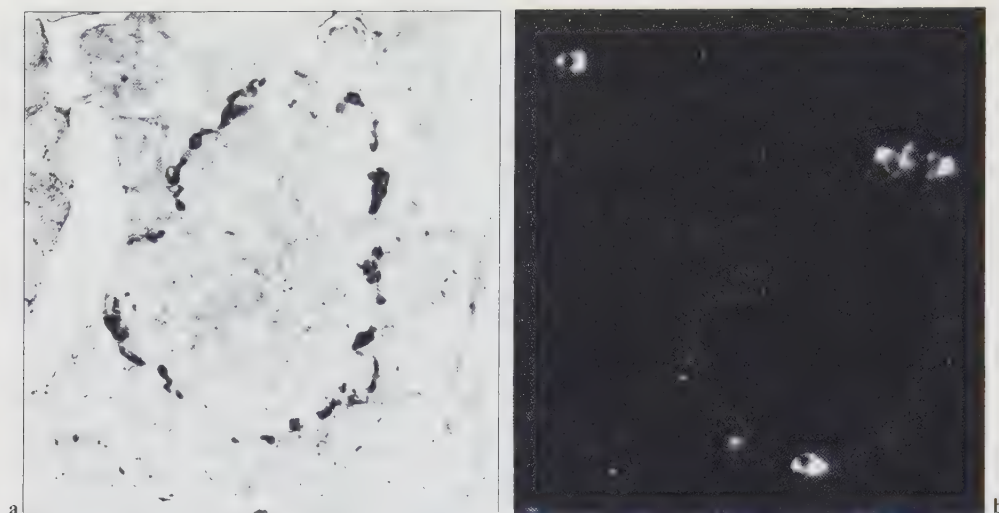


Fig. 3 a and b. Somatostatin immunohistochemistry. **a** Rat pancreas, duodenal portion, formaldehyde fixation. Immunoreactive cells in the islet periphery. $\times 200$. **b** Human pancreas duodenal portion, formaldehyde fixation. Somatostatin cells in duct epithelium and exocrine parenchyma. $\times 300$

Table 2. Extra-insular versus insular distribution of somatostatin cells in pancreas

	Insular	Extra-insular
Chicken	+	+
Rat	+	—
Rabbit	+	—
Cat	+	+
Dog	+	+
Pig	+	—
Man	+	+

could be detected either in the pancreas or in the gut wall. No immunoreactive cells could be detected within the thyroid.

In the chicken, somatostatin cells were fairly numerous in the proventriculus (Fig. 4a), absent from the gizzard, abundant in the gizzard-duodenal junction (antrum) (Fig. 4b), infrequent in the duodenum (Fig. 4c) and virtually absent from the remainder of the intestines. In the antrum they were found to predominate in the basal half of the mucosa, but scattered cells were also found in the upper portion. In the intestines they appeared to have a random topographic distribution. In the pancreas somatostatin cells predominated in the splenic lobe where they were very numerous, forming strands or columns in the center of the lobe (Fig. 5a). In the other parts of the pancreas the cells were much less numerous (Fig. 5b). Here they were found in islets as well as scattered in the exocrine parenchyma. On the whole, somatostatin cells seemed to be more numerous in the gut and pancreas of the chicken than of the mammals examined. As in mammals, no immunoreactive nerves could be detected in the gut wall and no immunoreactive cells in the thyroid or ultimobranchial gland.

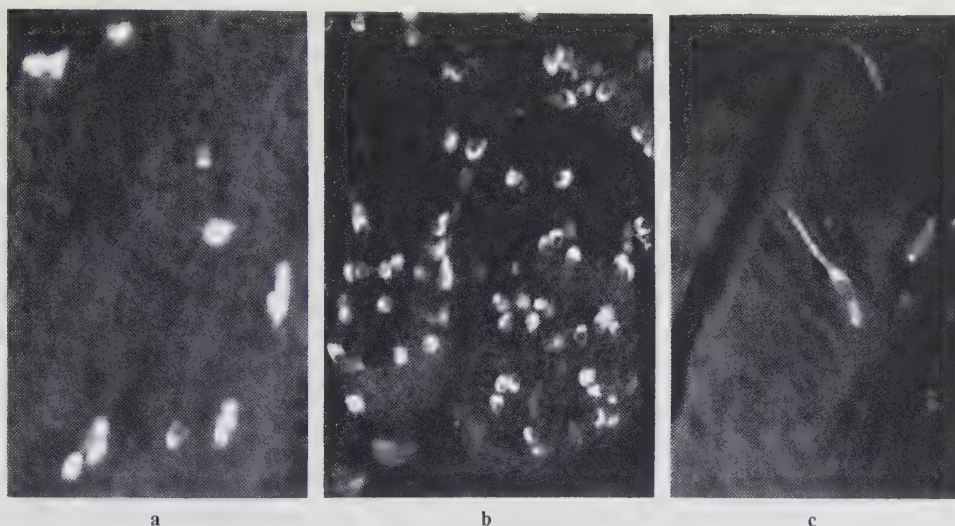


Fig. 4a-c. Somatostatin immunofluorescent cells in chicken gut. Formaldehyde fixation. **a** Proventriculus, $\times 300$. **b** Antrum, $\times 200$. **c** Duodenum, $\times 300$

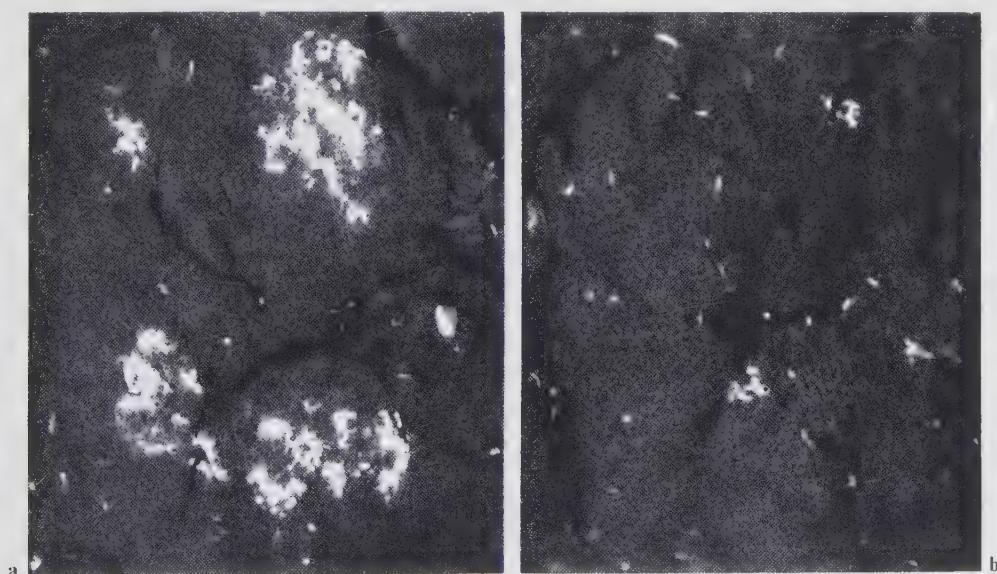


Fig. 5a and b. Somatostatin immunofluorescent cells in chicken pancreas. **a** Splenic lobe, DEPC fixation, $\times 150$. **b** Ventral lobe, formaldehyde fixation. $\times 150$

Somatostatin cells in the pancreas have been claimed to contain gastrin-immunoreactive material as well (Erlandsen et al., 1976; see also Lomsky et al., 1969; Greider and McGuigan, 1971). However, gastrin-immunoreactive cells could not be detected in the pancreas of the chicken, rat or man. In the cat and dog, a few gastrin-immunoreactive cells (much fewer than the somatostatin cells) occurred in the parenchyma of the duodenal lobe. As shown in studies on the chicken antrum,

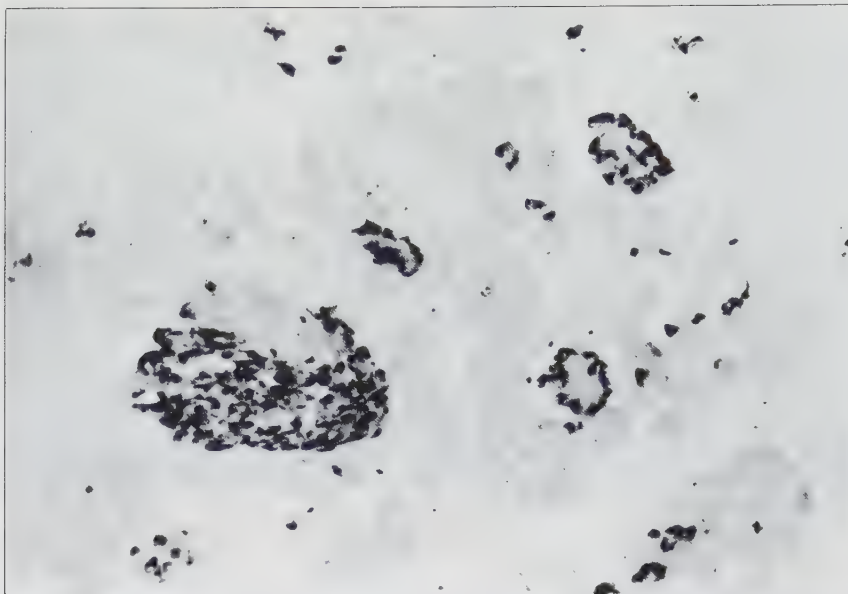
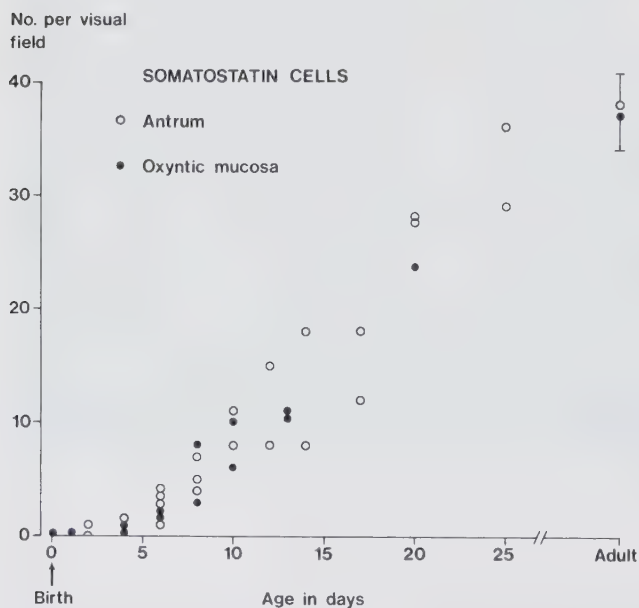


Fig. 6. Somatostatin immunohistochemistry. Pancreas of human fetus, 23 weeks gestational age. Numerous somatostatin cells in islet-like formations (PAP staining). $\times 175$



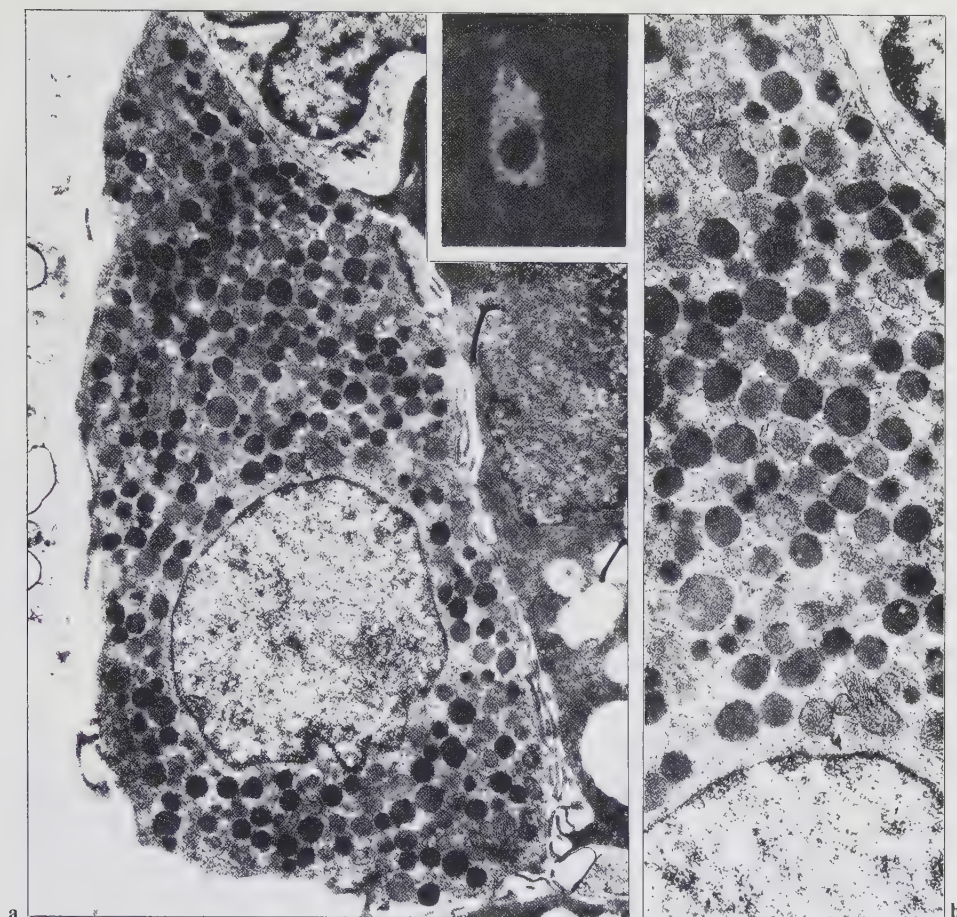


Fig. 8a and b. Immunocytochemical identification of somatostatin cells in cat antrum; glutaraldehyde-formaldehyde-osmium tetroxide fixation, resin embedding. Consecutive semithin/thin technique. *Insert:* Immunofluorescent cell in semithin section. The electron micrograph (a) demonstrates the fine structure of the same cell in an adjacent thin section. $\times 8800$. Higher magnification (b) to show granule ultrastructure. $\times 16,000$

Table 3. Diameter (nm) of granules in somatostatin cells of pancreas and stomach

Species	Pancreas	Stomach
Chicken	272 ± 70 (521)	287 ± 78 (1028)
Rat	170 ± 40 (548)	155 ± 39 (569)
Cat	246 ± 57 (583)	263 ± 64 (813)
Man	235 ± 45 (833)	240 ± 48 (418)

Mean $\pm$ S.D. (n)

gastrin cells in the gut are distinct from the somatostatin cells (cf. Sundler et al., 1977a).

Ontogeny of the Somatostatin Cells. In the human fetus somatostatin cells were numerous in the pancreas, stomach and small and large intestines at the 15th week

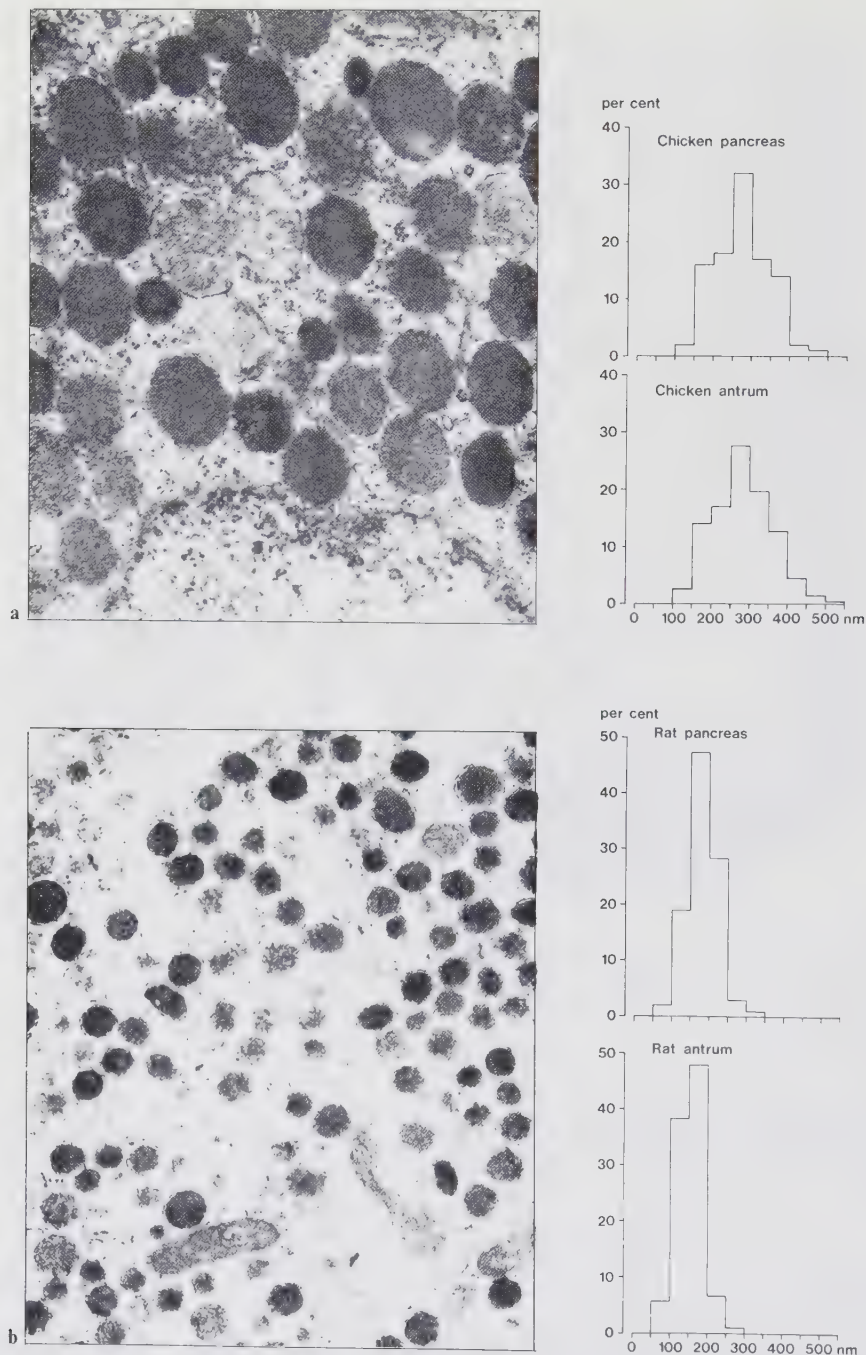
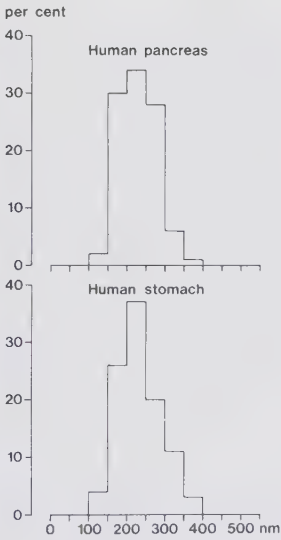
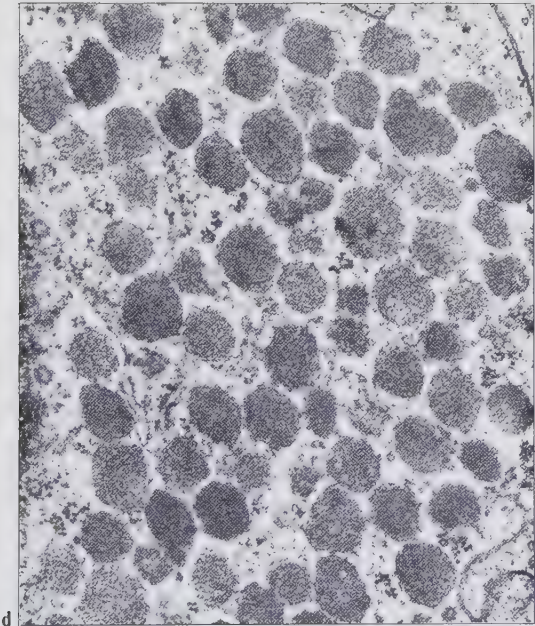
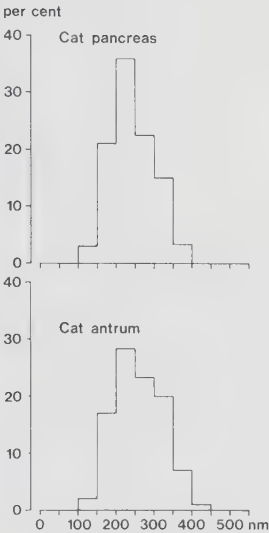
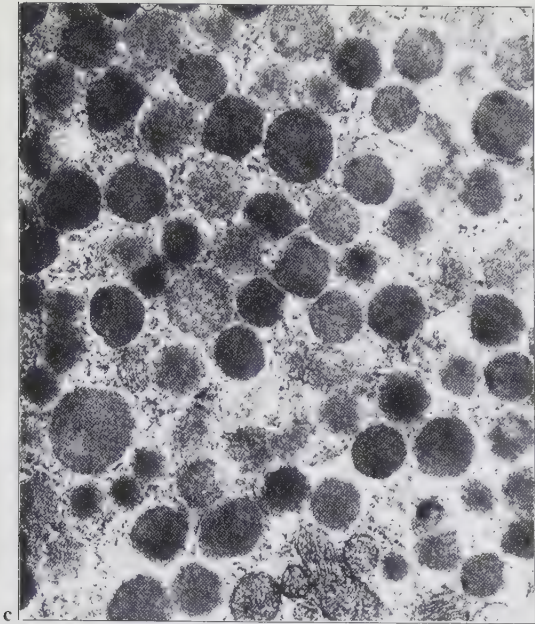


Fig. 9 a-d. Size distribution histograms (for details see Table 3) of somatostatin granules in cells from pancreas and gut of **a** chicken, **b** rat, **c** cat, **d** man, together with electron micrographs demonstrating granule ultrastructure in cells identified as somatostatin-storing by the consecutive semithin/thin section technique. $\times 30,000$. Note the small granule diameter in the somatostatin cell of the rat



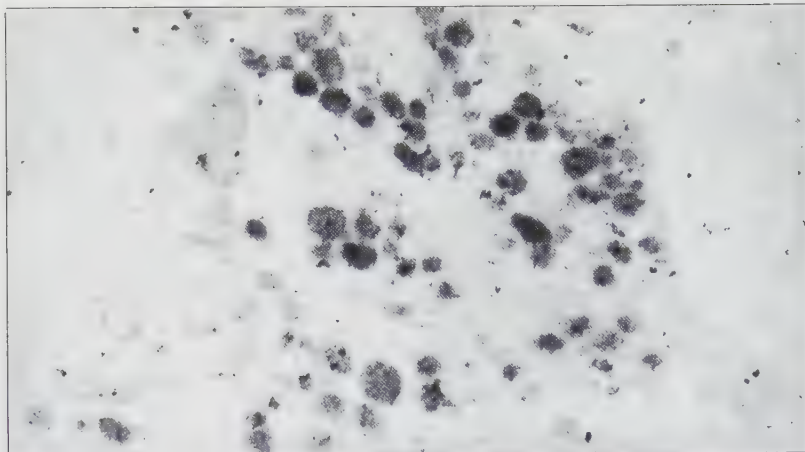


Fig. 10. Cat antrum; glutaraldehyde-formaldehyde-osmium tetroxide fixation. PAP staining of somatostatin cell, following treatment with hydrogen peroxidase and periodic acid in order to remove resin and osmium precipitates. The cytoplasmic granules are stained indicating the presence of somatostatin. $\times 15,000$

gestational age (earliest stage examined). Their number was further increased four to eight weeks later (Fig. 6).

In the fetal rat, somatostatin cells first appeared in the pancreas and duodenum (at the 16–17th day of gestation). Scattered immunoreactive cells were found in the remainder of the small intestine immediately before birth. In the gastric mucosa, somatostatin cells were first seen a few days after birth; they remained few during the first ten days of extra-uterine life (Fig. 7). Not until after about three weeks did they become as numerous as in the adult rat. One week after birth somatostatin cells were still very numerous in the pancreas, much more numerous (per unit area) than in the adult rat. Three weeks after birth somatostatin cells in the stomach and pancreas showed the adult frequency of occurrence and pattern of distribution.

In the chick embryo, somatostatin cells were first seen in the pancreas and proventriculus at about the 12th day of incubation. Not until about the 16th day of incubation were they observed in the duodenum. They were quite numerous in the latter location at the 18th day; in the proventriculus they formed clusters or nests at this stage. At about the 19th day they were detected in the antrum and one day later they occurred in the small intestine. At hatching and a few days later, somatostatin cells were very numerous in the pancreas. Near-adult numbers were found in the proventriculus, antrum and duodenum, whereas in the remainder of the small intestine and colon only few cells were detected.

Ultrastructure of Somatostatin Cells. The ultrastructure of the somatostatin cells in the chicken, rat, cat and man was defined using the consecutive semithin/ultrathin section technique: one section was used for immunohistochemical analysis and the consecutive section for electron microscopy (Fig. 8). The somatostatin cells were found to contain numerous round cytoplasmic granules which displayed a varying, often low, electron density and a fine-granulated texture. The limiting membrane

was tightly applied to the core. Their mean diameter was the same regardless of whether the cells examined were from the pancreas or from the gut, but the diameter differed greatly from one species to another. The somatostatin granules were largest in the chicken and smallest in the rat. These results are summarized in Table 3 and Fig. 9.

Immunocytochemistry (performed directly on ultrathin sections) showed that somatostatin is stored in the cytoplasmic granules (Fig. 10).

Discussion

Somatostatin cells are numerous in the pancreas and the upper digestive tract (for references, see Introduction). This observation was confirmed in all species examined. Particularly numerous are the somatostatin cells in the pancreas and antrum of the chicken (for a preliminary report on the distribution of somatostatin cells in the chicken gut, see Dubois, 1976; Larsson et al., 1976a). Contrary to the report by Leclerc et al. (1976) we found somatostatin cells of the open type in the antrum and the intestines. Erlandsen et al. (1976) reported that in rat, gastrin and somatostatin occurred conjunctively in the same cell in the pancreatic islets. In contrast, we have found gastrin-immunoreactive cells to be absent from the pancreas of the adult rat (see also Larsson et al., 1976b). Gastrin cells were also absent from the pancreas of the chicken and man. They were present in very low numbers in the duodenal portion of the exocrine pancreatic parenchyma of the cat and dog. Here they were much fewer than the somatostatin cells. It is therefore unlikely that gastrin and somatostatin reside in the same cell. We ascribe the difference in results to cross-reactivity of the anti-gastrin serum employed by Erlandsen et al. (1976).

Hökfelt et al. (1975a, b) detected somatostatin nerves in the rat gut, and Hökfelt et al. (1975a) and Parsons et al. (1976) described somatostatin immunoreactive cells in the thyroid. We were unable to confirm their observations. We ascribe these discrepancies to the presence of closely related, cross-reactive peptides which are detected by some anti-somatostatin sera but not by others.

The somatostatin cell was identified in the electron microscope as the D cell previously characterized by Caramia (1963) and Munger et al. (1965). Immunocytochemistry confirmed that somatostatin is stored exclusively in the cytoplasmic granules of this cell type (see also Leclerc et al., 1976). Except for the size of the granules, the ultrastructure of the somatostatin cells was similar in all species examined. The somatostatin granules were conspicuously small in the rat and quite large in the chicken. Regardless of whether the cells occurred in the pancreas or in the gut their ultrastructure was the same. The identification of the somatostatin cell in the chicken antrum was complicated by the abundance of other endocrine cell types in this region. Among these are gastrin cells (Larsson et al., 1974) and neurotensin cells (Sundler et al., 1977a, b), from which the somatostatin cells could be distinguished by the use of the consecutive semithin/ultrathin section technique.

In the developing rat and chicken, somatostatin cells appeared first in the pancreas and later in the digestive tract. They appeared very late in the stomach of

the rat, not until a few days after birth. Somatostatin cells appear in the digestive tract of the human fetus as early as the 10th week of gestation (Dubois et al., 1976). They were found to be very numerous at the 19–23rd week of gestation.

Orci and Unger (1975) reported the somatostatin cells to be interposed between the insulin cells and the glucagon cells and ascribed a special functional significance to the anatomical proximity between glucagon and somatostatin cells. It may be argued, however, that in certain species (chicken, cat and dog) somatostatin cells in the pancreas are quite numerous outside the islets. Moreover, Helmstaedter et al. (1976) have pointed out that in the pancreas of the horse somatostatin cells are peripherally located in the islets, whereas the glucagon cells are in the center, the insulin cells being interposed between the glucagon and somatostatin cells. Viewed in this perspective, it appears unlikely that there is a special functional relationship between the somatostatin cells and the glucagon cells. Whether or not this concept will eventually turn out to be correct, the functional role of the somatostatin cells in the gut remains to be established.

Acknowledgements. Grant support from the Swedish Medical Research Council (04X-4477), Pålsson's Foundation and the Swedish Diabetes Association.

References

- Arimura, A., Sato, H., Dupont, A., Nishi, N., Schally, A.V.: Somatostatin: Abundance of immunoreactive hormone in rat stomach and pancreas. *Science* **189**, 1007–1009 (1975)
- Beauvillain, J.-C., Tramu, G., Dubois, M.P.: Characterization by different techniques of adrenocorticotropin and gonadotropin producing cells in Lerot pituitary (*Eliomys quercinus*). *Cell Tiss. Res.* **158**, 301–317 (1975)
- Björklund, A., Falck, B., Owman, Ch.: Fluorescence microscopic and microspectro-fluorometric techniques for the cellular localization and characterization of biogenic amines. In: *Methods of investigative and diagnostic endocrinology*, edit. S.A. Berson, Vol I: The thyroid and biogenic amines, edit. by J.E. Rall and I.J. Kopin, pp. 318–368. Amsterdam: North-Holland Publ. Comp. 1972
- BrazEAU, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., Guillemin, R.: Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179**, 77–79 (1973)
- Caramia, F.: Electron microscopic description of a third cell type in the islets of the rat pancreas. *Amer. J. Anat.* **112**, 53–64 (1963)
- Dubois, M.P.: Presence of immunoreactive somatostatin in discrete cells of the endocrine pancreas. *Proc. nat. Acad. Sci. (Wash.)* **72**, 1340–1343 (1975)
- Dubois, M.P.: Le système à somatostatine. *Ann. Endocrinol. (Paris)* **37**, 277–278 (1976)
- Dubois, P.M., Paulin, C., Assan, R., Dubois, M.P.: Evidence for immunoreactive somatostatin in the endocrine cells of human foetal pancreas. *Nature (Lond.)* **256**, 731–732 (1975)
- Dubois, P.M., Paulin, C., Dubois, M.P.: Gastrointestinal somatostatin cells in the human foetus. *Cell Tiss. Res.* **166**, 179–184 (1976)
- Erlandsen, S.L., Hegre, O.D., Parsons, J.A., McEvoy, R.C., Elde, R.P.: Pancreatic islet cell hormones: distribution of cell types in the islet and evidence for the presence of somatostatin and gastrin within the D cell. *J. Histochem. Cytochem.* **24**, 883–897 (1976)
- Goldsmith, P.C., Rose, J.C., Arimura, A., Ganong, W.F.: Ultrastructural localization of somatostatin in pancreatic islets of the rat. *Endocrinology* **97**, 1061–1064 (1975)
- Greider, M.H., McGuigan, J.E.: Cellular localization of gastrin in the human pancreas. *Diabetes* **20**, 389–396 (1971)
- Helmstaedter, V., Feurle, G.E., Forssmann, W.G.: Insulin-, glucagon-, and somatostatin-immunoreactive endocrine cells in the equine pancreas. *Cell Tiss. Res.* **172**, 447–454 (1976)
- Hököfelt, T., Efendic, S., Hellerström, C., Johansson, O., Luft, R., Arimura, A.: Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special reference to the A₁-cells of the pancreatic islets and to the hypothalamus. *Acta endocr. (Kbh.), Suppl.* 1–41 (1975a)

- Hökfelt, T., Johansson, O., Efendic, S., Luft, R., Arimura, A.: Are there somatostatin-containing nerves in the rat gut? Immunohistochemical evidence for a new type of peripheral nerves. *Experientia* (Basel) **31**, 852–853 (1975b)
- Larsson, L.-I., Rehfeld, J.F., Sundler, F., Håkanson, R.: Pancreatic gastrin in foetal and neonatal rats. *Nature* (Lond.) **262**, 609–610 (1976b)
- Larsson, L.-I., Sundler, F., Håkanson, R.: Pancreatic hormones in the gut and gut hormones in the pancreas. In: *Endocrine gut and pancreas*. Ed. T. Fujita, pp. 133–143. Amsterdam: Elsevier 1976a
- Larsson, L.-I., Sundler, F., Håkanson, R., Rehfeld, J.F., Stadil, F.: Distribution and properties of gastrin cells in the gastrointestinal tract of chicken. *Cell Tiss. Res.* **154**, 409–421 (1974)
- Leclerc, R., Pelletier, G., Puviani, R., Arimura, A., Schally, A.V.: Immunohistochemical localization of somatostatin in endocrine cells of the rat stomach. *Mol. Cell. Endocrinol.* **4**, 257–261 (1976)
- Lomsky, R., Langr, F., Vortel, V.: Immunohistochemical demonstration of gastrin in mammalian islets of Langerhans. *Nature* (Lond.) **223**, 618–619 (1969)
- Luft, R., Efendic, S., Hökfelt, T., Johansson, O., Arimura, A.: Immunohistochemical evidence for the localization of somatostatin-like immunoreactivity in a cell population of the pancreatic islets. *Med. Biol.* **52**, 428–430 (1974)
- Mayor, H.D., Hampton, J.C., Rosario, R.: A simple method for removing the resin from epoxy-embedded tissue. *J. biophys. Cytol.* **9**, 909–910 (1961)
- Munger, B.L., Caramia, F., Lacy, P.E.: The ultrastructural basis for the identification of cell types in the pancreatic islets. II. Rabbit, dog and opossum. *Z. Zellforsch.* **67**, 776–798 (1965)
- Orci, L., Baetens, D., Dubois, M.P., Rufener, C.: Evidence for the D-cell of the pancreas secreting somatostatin. *Horm. Metab. Res.* **7**, 400–405 (1975)
- Orci, L., Baetens, D., Ravazzola, M., Malaisse-Lagae, F., Amherdt, M., Rufener, C., Somatostatin in the pancreas and the gastrointestinal tract. In: *Endocrine gut and pancreas*. Ed. T. Fujita, pp. 73–88. Amsterdam: Elsevier 1976
- Orci, L., Unger, R.: Functional subdivision of islets of Langerhans and possible role of D cells. *Lancet* **1975**, 1233–1234
- Parsons, J.A., Erlandsen, S.L., Hegre, O.D., McEvoy, R.C., Elde, R.P.: Central and peripheral localization of somatostatin. Immunoenzyme immunocytochemical studies. *J. Histochem. Cytochem.* **24**, 872–882 (1976)
- Pearse, A.G.E., Polak, J.M.: Bifunctional reagents as vapour- and liquid-phase fixatives for immunohistochemistry. *Histochem. J.* **7**, 179–186 (1975)
- Pelletier, S., Leclerc, R., Arimura, A., Schally, A.V.: Immunohistochemical localization of somatostatin in the rat pancreas. *J. Histochem. Cytochem.* **23**, 699–701 (1975)
- Polak, J.M., Pearse, A.G.E., Grimelius, L., Bloom, S.R., Arimura, A.: Growth hormone release-inhibiting hormone in gastrointestinal and pancreatic D-cells. *Lancet* **1975**, 1220–1222
- Rehfeld, J.F., Stadil, F., Rubin, B.: Production and evaluation of antibodies for the radioimmunoassay of gastrin. *Scand. J. clin. Lab. Invest.* **30**, 221–232 (1972)
- Rufener, C., Amherdt, M., Dubois, M.P., Orci, L.: Ultrastructural immunocytochemical localization of somatostatin in D-cells of rat pancreatic monolayer culture. *J. Histochem. Cytochem.* **23**, 966–969 (1975c)
- Rufener, C., Dubois, M.P.: Cells immunofluorescent to anti-growth hormone release inhibiting factor (somatostatin) in endocrine pancreas and digestive tract. *Diabetologia* **11**, 373 (1975a) (abs.)
- Rufener, C., Dubois, M.P., Malaisse-Lagae, F., Orci, L.: Immunofluorescent reactivity to anti-somatostatin in the gastrointestinal mucosa of the dog. *Diabetologia* **11**, 321–324 (1975b)
- Sternberger, L.A.: *Immunocytochemistry*. New Jersey: Prentice-Hall, Inc. 1974
- Sundler, F., Alumets, J., Håkanson, R., Carraway, R., Leeman, S.E.: Ultrastructure of the gut neurotensin cell. *Histochemistry* **53**, 25–34 (1977a)
- Sundler, F., Håkanson, R., Hammer, R.A., Alumets, J., Carraway, R., Leeman, S.E., Zimmerman, E.: Immunohistochemical localization of neurotensin in endocrine cells of the gut. *Cell. Tiss. Res.* **178**, 313–321 (1977b)
- Weir, G.C., Goltsof, P.C., Sternberg, E.P., Patel, Y.C.: High concentration of somatostatin immunoreactivity in chicken pancreas. *Diabetologia* **12**, 129–132 (1976)

Leu-Enkephalin-Like Material in Nerves and Enterochromaffin Cells in the Gut

An Immunohistochemical Study

J. Alumets, R. Håkanson, F. Sundler, and K.-J. Chang

Departments of Histology and Pharmacology, University of Lund, Lund, Sweden
and Molecular Biology Department, The Wellcome Research Laboratories,
Burroughs Wellcome Co., Research Triangle Park, N.C., U.S.A.

Summary. The distribution and cellular localization of leu-enkephalin in the gut and pancreas was studied by immunohistochemistry using two different antisera, one specifically directed against leu-enkephalin and the other cross reacting with met-enkephalin. The results were identical with both antisera. In all species examined, enkephalin-immunoreactive material was found in nerves of the smooth muscle, particularly numerous in the myenteric plexus. Here, immunoreactive nerve cell bodies were observed occasionally. In addition, enkephalin-immunoreactive material was demonstrated in gut endocrine cells of chicken, mouse, rat, pig and monkey but not of guinea pig, cat and man. Enkephalin cells were detected also in the exocrine parenchyma of the porcine pancreas. They were rare in the gut of mouse, rat and monkey but numerous in the antrum and duodenum of pig where they were identified as 5-hydroxytryptamine-storing enterochromaffin cells. The enkephalin-containing cells of the porcine antrum and duodenum were defined ultrastructurally by the consecutive semithin/ultrathin section technique. The ultrastructural features were typical of enterochromaffin cells, the most characteristic ones being the irregular shape and high electron density of the cytoplasmic granules. The immunoreactive material was confined to the cytoplasmic granules.

Introduction

Enkephalin, a peptide consisting of five amino acids, was first isolated from porcine brain by virtue of its potent opiate receptor agonist activity (Hughes et al., 1975). The peptide was found to occur in two varieties; one having leucine in C-terminal position (leu-enkephalin), the other having methionine (met-enkephalin). Several recent studies have shown the enkephalins to be members of a whole family of chemically related peptides— β -lipotropin and the endorphins—all of which contain the met-enkephalin 5 amino acid sequence (Goldstein, 1976; Lazarus et al., 1977). Both β -lipotropin and the endorphins

seem to occur in large amounts in the brain (Elde et al., 1976; Hökfelt et al., 1977; Rossier et al., 1977; Simantov et al., 1977; Watson et al., 1977; Yang et al., 1977); the larger members of the family (β -lipotropin, α -, β - and γ -endorphins) are present also in the adenohypophysis where they are located in the ACTH and MSH cells (Moon et al., 1973; Furth et al., 1975; Bloom et al., 1977; Pelletier et al., 1977).

It is well known that morphine receptors are present in the gut (cf. Kosterlitz et al., 1973; Creese and Snyder, 1975). Not unexpectedly therefore, large amounts of endogenous opiate receptor ligands were demonstrated in the gut wall, and found to be releasable upon electric stimulation (Puig et al., 1977; Schulz et al., 1977). Elde et al. (1976) and Polak et al. (1977) have recently reported on the presence of nerves displaying enkephalin immunoreactivity in the myenteric plexus. In addition, Polak et al. (1977) found enkephalin-immunoreactive material in the gastrin cells of the human antrum. The present report deals with the occurrence and distribution of leu-enkephalin-immunoreactive material in the gut of several species, including man.

Material and Methods

Antisera. We had access to two rabbit antisera against leucine-enkephalin. One was raised by one of us (K.-J. C., Burroughs Wellcome) using synthetic leu-enkephalin conjugated with bovine serum albumin (Miller et al., 1978). The other (code no 6-P3) was a generous gift from Miles-Yeda and the immunization protocol is unknown to us. Fluoresceinated as well as unlabelled sheep anti-rabbit IgG was purchased from SBL, Stockholm, Sweden. Peroxidase-antiperoxidase (PAP) complex was obtained from Cappel Labs., Downington, Pa., U.S.A.

Chemicals. Opiate peptides were purchased from UCB, Brussels, Belgium, from Peninsula, San Carlos, Calif. or from Bachem, Torrance, Calif. Leu-enkephalin was purchased from Bachem. The following opiate peptides were tested from cross reactivity with the antisera: β -Lipotropin 61-91 (β -endorfin; Peninsula), β -Lipotropin 60-65 (Peninsula), β -Lipotropin 61-76 (α -endorfin; Bachem), β -Lipotropin 61-77 (γ -endorfin; UCB), β -Lipotropin 61-65 (met-enkephalin, Bachem).

Immunohistochemistry. Chicken, mouse, rat, guinea-pig, rabbit, cat, pig, monkey and man were studied. Chickens were killed by decapitation. Mice, rats and guinea-pigs were killed by exsanguination under diethyl ether anesthesia. Rabbits were killed by the i.v. injection of air. Cats and monkeys were killed by an overdose of sodium pentobarbital (Nembutal®). Porcine material was obtained immediately after death at a local slaughter-house. Human material was obtained at abdominal surgery (by courtesy of Drs. S. Ingemansson and G. Liedberg, Department of Surgery, University Hospital, Lund, Sweden). Specimens were collected from various locations along the gut and from the pancreas. They were frozen to the temperature of liquid nitrogen in a propane-propylene mixture and freeze-dried. The specimens were then vapour-fixed with diethylpyrocarbonate (Pearse and Polak, 1975) or formaldehyde (Björklund et al., 1972) and embedded in paraffin or Epon. Sections were cut at 5 μ m (paraffin) or 1 μ m (Epon). After removal of the embedding medium the sections were carried down to water in graded ethanol solutions and then processed for the immunohistochemical demonstration of enkephalin using the indirect immunofluorescence procedure (Coons et al., 1955) or the PAP technique (cf. Sternberger, 1974). For working dilutions of the two enkephalin antisera see Table 1. As controls served sections incubated with the two antisera inactivated by addition of the antigen in excess. For evaluation of cross reactivity each of the various opiate peptides were added to the antisera in amounts from 1.0 to 100 μ g per ml diluted serum. Sections processed for immunofluorescence were examined in a fluorescence microscope equipped with an epi-illumination system. Filters were selected to give peak excitation at 490 nm. For examination of formaldehyde-induced fluorescence, filters were selected to give peak excitation at 410 nm.

Table 1. Working dilutions of the leu-enkephalin antisera

Antisera:	Burroughs-Wellcome		Miles-Yeda	
Detection:	FITC	PAP	FITC	PAP
Fixation				
Formaldehyde	1:60	1:240	1:60	1:160
Diethylpyrocarbonate	1:60	1:240		1:640

FITC = Immunofluorescence; PAP = Immunoperoxidase

Electron Microscopy and Immunocytochemistry. Small pieces were cut out from porcine antral and duodenal mucosa and fixed by immersion in 1% glutaraldehyde and 3% formaldehyde in 0.075 M sodium phosphate buffer. The specimens were postfixed in 1% osmium tetroxide in the same buffer for one hour, dehydrated in graded ethanol solutions, stained with 1% phosphotungstic acid and 0.5% uranyl acetate in ethanol and embedded in Epon.

Identification of enkephalin immunoreactive cells at the ultrastructural level was made by the consecutive semithin/ultrathin section technique. Semithin ($<0.3 \mu$) sections were mounted on glass slides. The plastic was removed with sodium methoxide according to Mayor et al. (1961). Osmium deposits were removed with 1% periodic acid (see Beauvillain et al., 1975). The sections were then processed for the immunohistochemical demonstration of enkephalin (see above). Adjacent ultrathin sections were placed on grids and contrasted with lead citrate and uranyl acetate and examined in a Philips EM 300 electron microscope. In one experiment enkephalin immunoreactivity was demonstrated by PAP staining of ultrathin sections (for details see Beauvillain et al., 1975). Granule size was established by measuring the diameter of all cytoplasmic granule profiles in 10 identified cells from porcine antrum and duodenum. In pleiomorphic granules the smallest and largest diameter were measured and the diameter given as the mean. The photomicrographs were reproduced at a magnification of $15,000\times$.

Results

Enkephalin immunoreactive nerves were present in the gut wall of all species examined; the immunoreactivity was equally well preserved following either diethylpyrocarbonate or formaldehyde fixation. The immunoreactive nerves were particularly prominent in the guinea-pig. The myenteric plexus all along the gut contained a dense network of enkephalin fibres which were seen to surround non-reactive cell bodies (Fig. 1). Scattered immunoreactive nerves were observed also in the smooth muscle layer outside the plexus and in the lamina muscularis mucosae (Fig. 2). Only occasionally were enkephalin nerves seen in the submucosa. In no species and in no segment of the gut were enkephalin nerves observed in the mucosa. The density of immunoreactive nerves was fairly uniform all along the gut. In the mouse, guinea-pig and monkey single or clustered immunoreactive nerve cell bodies were encountered within the myenteric plexus (Fig. 3). These observations were identical with both antisera.

Immunoreactive endocrine cells were seen in the gut of chicken, mouse, rat, pig and monkey (Fig. 4). Also these findings were the same with both antisera. In the chicken the immunoreactive cells occurred in the small intestine, the caeca and the colon. In the rabbit immunoreactive cells occurred basally in the gastric glands. In the mouse, rat and monkey immunoreactive cells were

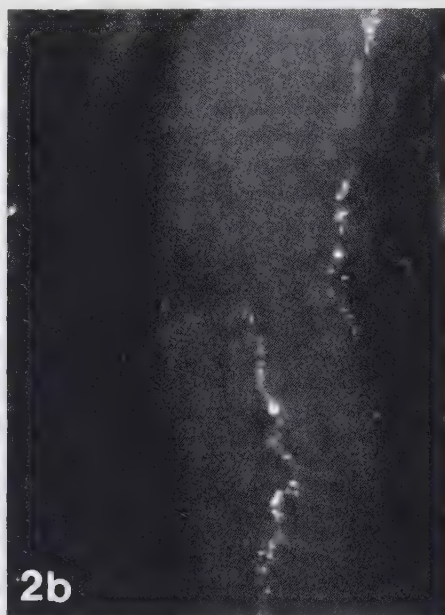
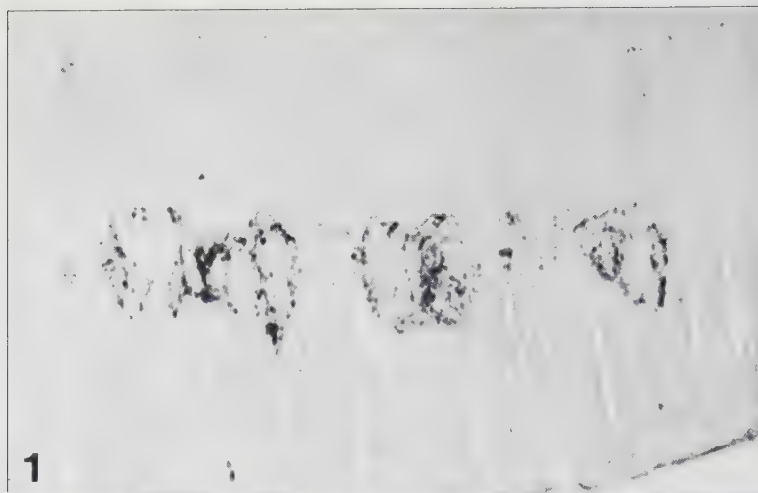


Fig. 1. Mouse, colon, formaldehyde fixation, PAP staining. Immunoreactive nerve terminals in the myenteric plexus, surrounding non-reactive nerve cell bodies. $\times 200$

Fig. 2. a Homo, jejunum, diethylpyrocarbonate fixation. Immunofluorescent nerves in the lamina muscularis externa. $\times 250$. **b** Guinea pig, jejunum, formaldehyde fixation. Immunoreactive nerves in the lamina muscularis mucosae. $\times 300$

restricted to the antrum where they were few, much fewer than e.g. the gastrin or somatostatin cells. In the pig enkephalin immunoreactive endocrine cells were numerous in the antrum and proximal duodenum. Few scattered enkephalin cells were detected also in the exocrine parenchyma of the porcine pancreas. In the porcine antrum the enkephalin-immunoreactive endocrine cells were found

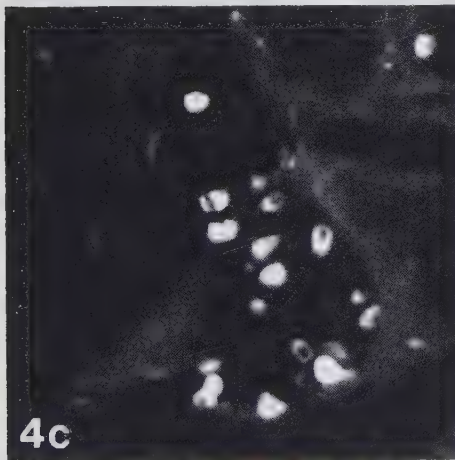
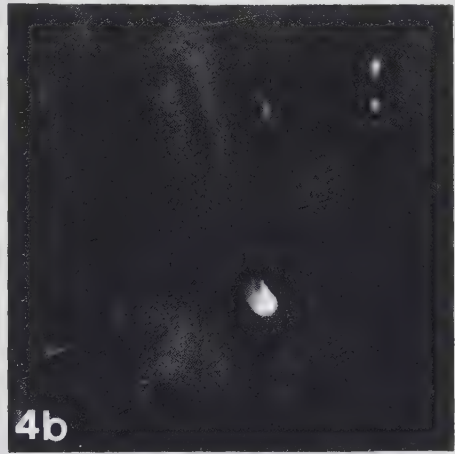
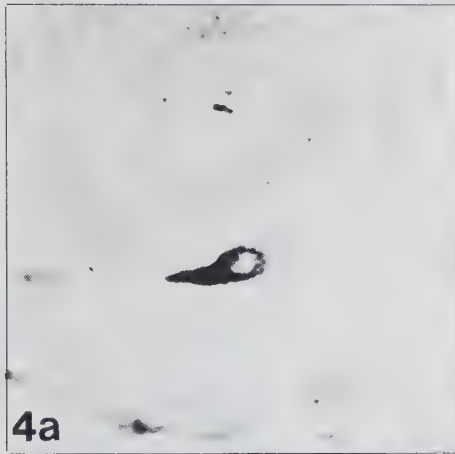
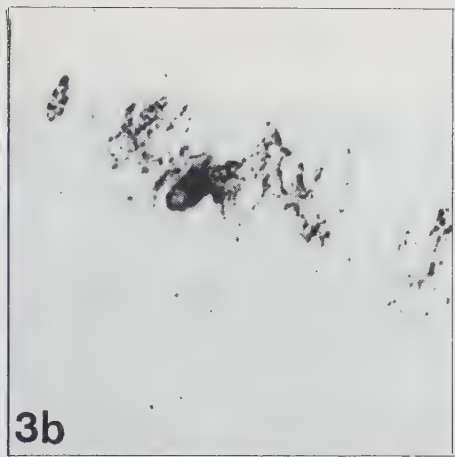
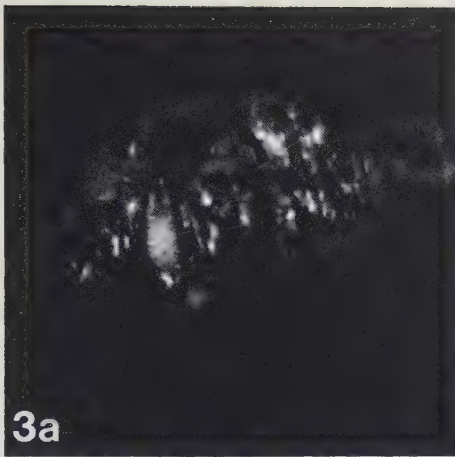


Fig. 3. **a** Cat, ileum, formaldehyde fixation. Numerous nerve fibers and a single nerve cell body displaying enkephalin-immunofluorescence in the myenteric plexus. $\times 200$. **b** Mouse, colon, diethylpyrocarbonate fixation. Single immunoreactive nerve cell body in the myenteric plexus. PAP staining. $\times 200$

Fig. 4a–d. Immunoreactive endocrine cells in the digestive tract of several species. **a** Chicken, small intestine. Single immunoreactive endocrine cell basally in crypt epithelium. PAP staining. $\times 400$. **b** Mouse, antrum. Single immunofluorescent endocrine cell basally in pyloric gland. $\times 200$. **c** Pig, duodenum. Numerous immunofluorescent endocrine cells. $\times 150$. **d** Monkey, duodenum. Two endocrine cells displaying different intensity of immunofluorescence. $\times 400$

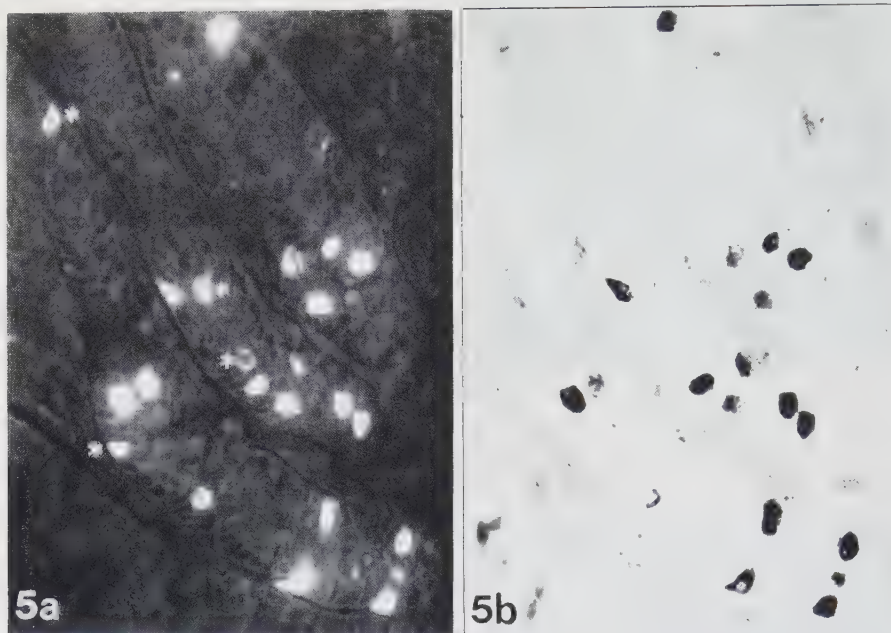


Fig. 5a and b. Pig, duodenum, formaldehyde fixation. **a** Formaldehyde-induced fluorescence in 5-HT-containing enterochromaffin cells. $\times 166$. **b** Same section processed for immunohistochemical demonstration of enkephalin. PAP staining. All immunoreactive cells are 5-HT-storing but some 5-HT-storing cells (asterisk) do not display enkephalin immunoreactivity. $\times 166$

Table 2. Specificity of anti-leu-enkephalin serum: The minimum amount ($\mu\text{g}/\text{ml}$) of various opiate peptides required to block the immunoreaction

Antiserum (source)	Burroughs-Wellcome $\mu\text{g}/\text{ml}$	Miles-Yeda (6 P3) $\mu\text{g}/\text{ml}$
Peptide		
Leu-enkephalin	1.0	1.0
Met-enkephalin	10	1.0
β -Lipotropin 60-65	10	1.0
α -Endorphin	100	10
γ -Endorphin	100	100
β -Endorphin	100	100

Sections of porcine antrum and guinea pig ileum (diethylpyrocarbonate fixation) were used. Peptides were tested in doses ranging from 1.0 to 100 μg per ml diluted serum

to be identical with 5-hydroxytryptamine-storing enterochromaffin cells. This was established by consecutive staining of one and the same section: demonstration of formaldehyde-fluorescent 5-hydroxytryptamine-storing enterochromaffin cells was followed by immunoperoxidase staining for enkephalin (Fig. 5). Approximately 70% of the enterochromaffin cells in the porcine antrum and proximal duodenum were stained. In the digestive tract of guinea pig, cat and man no immunoreactive endocrine cells were observed with either of the two enkephalin antisera. As shown in Table 2 the specificity of the two antisera differed in that antiserum 6-P3 showed also some cross reactivity with met-enkephalin.

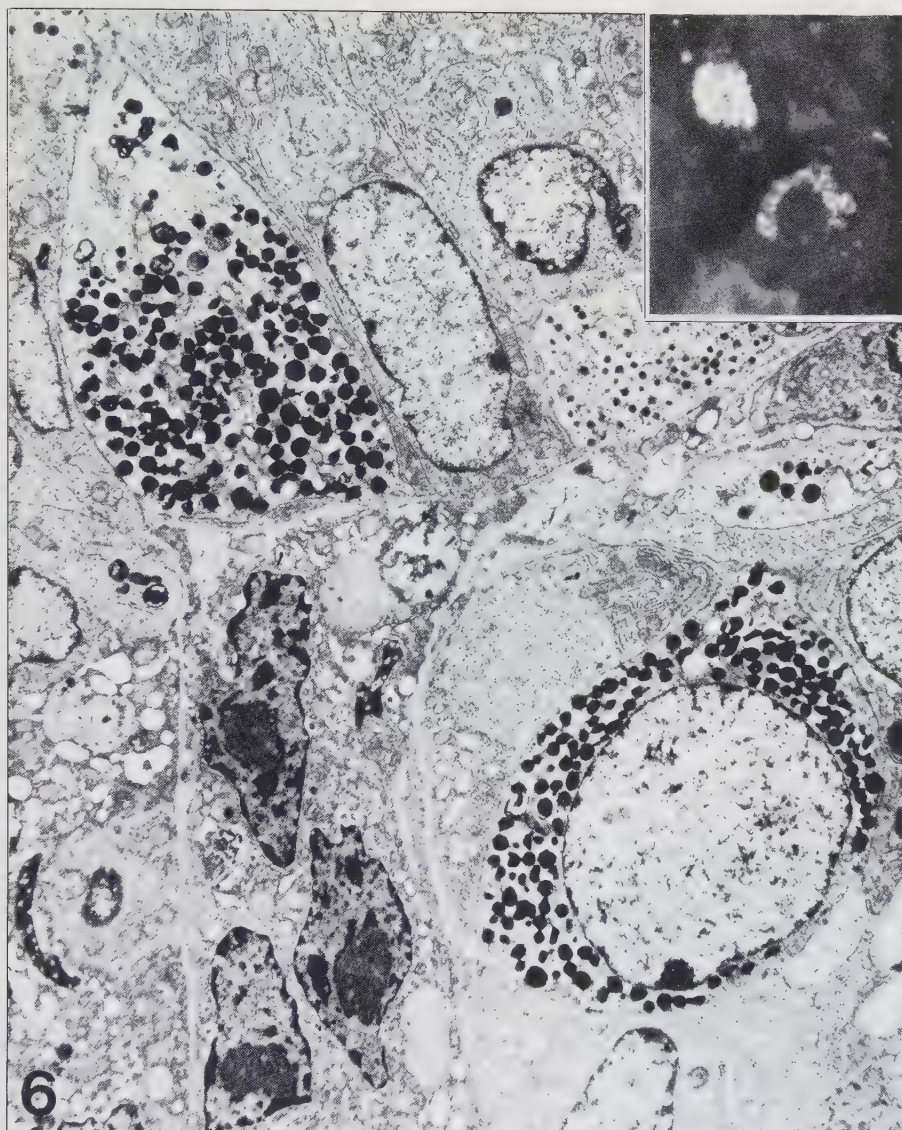


Fig. 6. Immunocytochemical identification of enkephalin cell in porcine antrum. Formaldehyde-glutaraldehyde-osmium tetroxide fixation, Epon. Consecutive semithin/ultrathin section technique. Insert: Two immunofluorescent enkephalin cells in semithin section. $\times 1000$. The electron micrograph demonstrates fine structural details in the same cells in an adjacent ultrathin section. $\times 6000$

With both antisera large amounts of β -endorphin were required to block the immunoreaction.

The enkephalin-immunoreactive cells in porcine gut were identified ultra-structurally by the consecutive semithin/ultrathin section technique (Fig. 6). They were characterized by the presence in their cytoplasm of numerous, fairly large,

- Creese, I., Snyder, S.H.: Receptor binding and pharmacological activity of opiates in the guinea-pig intestine. *J. Pharmacol. Exp. Ther.* **194**, 205-219 (1975)
- Elde, R., Hökfelt, T., Johansson, O., Terenius, L.: Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. *Neuroscience* **1**, 349-351 (1976)
- Furth, J., Chrétien, M., Lis, M., Belanger, A., Moy, P., Grauman, J.: Multipotent lipotropic hormones. *Arch. Pathol.* **99**, 572-582 (1975)
- Goldstein, A.: Opioid peptides (endorphins) in pituitary and brain. *Science* **193**, 1081-1086 (1976)
- Hökfelt, T., Elde, R., Johansson, O., Terenius, L., Stein, L.: The distribution of enkephalin-immunoreactive cell bodies in the rat central nervous system. *Neuroscience Lett.* **5**, 25-31 (1977)
- Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., Morris, H.R.: Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature (Lond)* **258**, 577-579 (1975)
- Kosterlitz, H.W., Lord, J.A.H., Watt, A.J.: Morphine receptor in the myenteric plexus of the guinea-pig ileum. In: *Agonist and Antagonist Actions of Narcotic Analgesic Drugs* (ed. H.W. Kosterlitz, H.O.S. Collier and J.E. Villarreal), pp. 45-61. Baltimore: University Park Press 1973
- Lazarus, L.H., Ling, N., Guillemin, R.: β -Lipotropin as a prohormone for the morphinomimetic peptides endorphins and enkephalins. *Proc. Natl. Acad. Sci. USA* **73**, 2156-2159 (1977)
- Mayor, H.D., Hampton, J.C., Rosario, R.: A simple method for removing the resin from epoxy-embedded tissue. *J. biophys. Cytol.* **9**, 909-910 (1961)
- Miller, R.J., Chang, K.-J., Cooper, B., Cuatrecasas, P.: Radioimmunoassay and characterization of enkephalins in rat tissues. *J. Biol. Chem.* **253**, 531-538 (1978)
- Moon, H.D., Li, C.H., Jennings, B.M.: Immunohistochemical studies of pituitary β -lipotrophs. *Anat. Rec.* **175**, 529-538 (1973)
- Nilsson, G., Larsson, L.-I., Håkanson, R., Brodin, E., Pernow, B., Sundler, F.: Localization of substance P-like immunoreactivity in mouse gut. *Histochemistry* **43**, 97-99 (1975)
- Pearse, A.G.E., Polak, J.M.: Bifunctional reagents as vapour- and liquid-phase fixatives for immunohistochemistry. *Histochem. J.* **7**, 179-186 (1975)
- Pelletier, G., Leclerc, R., Labrie, F., Côté, J., Chrétien, M., Lis, M.: Immunohistochemical localization of β -lipotropic hormone in the pituitary gland. *Endocrinology* **100**, 770-776 (1977)
- Polak, J., Sullivan, S.N., Bloom, S.R., Facer, P., Pearse, A.G.E.: Enkephalin-like immunoreactivity in the human gastrointestinal tract. *Lancet* **1977/I**, 972-974
- Puig, M.M., Gascon, P., Craviso, G.L., Musacchio, J.: Endogenous opiate receptor ligand: Electrically induced release in the guinea pig ileum. *Science* **195**, 419-420 (1977)
- Rossier, J., Vargo, T.M., Minick, S., Ling, N., Bloom, F.E., Guillemin, R.: Regional dissociation of β -endorphin and enkephalin contents in rat brain and pituitary. *Proc. Natl. Acad. Sci. USA* **74**, 5162-5165 (1977)
- Schulz, R., Wüster, M., Simantov, R., Snyder, S., Herz, A.: Electrically stimulated release of opiate-like material from the myenteric plexus of the guinea pig ileum. *Europ. J. Pharmacol.* **41**, 347-348 (1977)
- Simantov, R., Kuhar, M.J., Uhl, G.R., Snyder, S.H.: Opioid peptide enkephalin: Immunohistochemical mapping in rat central nervous system. *Proc. Natl. Acad. Sci. USA* **74**, 2167-2177 (1977)
- Sternberger, L.: *Immunocytochemistry*. Englewood Cliffs, N.J.: Prentice-Hall, Inc. 1974
- Sundler, F., Håkanson, R., Larsson, L.-I., Brodin, E., Nilsson, G.: Substance P in the gut: An immunohistochemical and immunohistochemical study of its distribution and development. In: *Substance P* (eds.: U.S. von Euler and B. Pernow) pp. 59-65. New York: Raven Press 1977
- Watson, S.H., Barchas, J.D., Li, C.H.: β -Lipotropin: Localization of cells and axons in rat brain by immunocytochemistry. *Proc. Natl. Acad. Sci. USA* **74**, 5155-5158 (1977)
- Yang, H.-Y., Hong, J.S., Costa, E.: Regional distribution of leu and met enkephalin in rat brain. *Neuropharmacol.* **16**, 303-307 (1977)

5-HYDROXYTRYPTAMINE-CONTAINING ENTEROCHROMAFFIN CELLS: STORAGE SITE OF SUBSTANCE P

F. Sundler, J. Alumets and R. Håkanson

Departments of Histology and Pharmacology, University of Lund, Lund, Sweden

Substance P was detected by von Euler and Gaddum (1) in extracts of brain and intestine by virtue of its potent hypotensive and gut smooth muscle stimulating effects. Recently, substance P was isolated and found to be an 11-amino acid peptide (2). By immunohistochemistry substance P has been found to have a dual distribution in the gut; in a system of intrinsic nerves and in endocrine-like cells scattered in the mucosa (3,4). From the distribution pattern of the immunoreactive endocrine-like cells it was suggested that they belonged to the system of enterochromaffin cells, known to be the storage site of 5-hydroxytryptamine (5-HT) (cf. 5). The recent finding of large amounts of substance P immunoreactive material in certain 5-HT-producing gut carcinoid tumours (6,7) supports this assumption. In order to get direct evidence for the occurrence of substance P immunoreactivity in enterochromaffin cells a study was performed on the mouse colon, known to be fairly rich in substance P immunoreactive cells (3). Specimens were dissected out and frozen in a propane-propylene mixture, cooled to the temperature of liquid nitrogen, and freeze-dried. Some specimens were exposed to diethylpyrocarbonate (DEPC) vapours for 3 hrs at 55°C (8), while others were treated with formaldehyde vapours for 1 hr at 80°C (standard Falck-Hillarp procedure (9). All specimens were embedded in paraffin in vacuo. Sections were cut at 5 µm and placed on albuminized glass slides.

Formaldehyde vapour fixation induces strong fluorescence from 5-HT (9) but preserves substance P immunoreactivity only poorly. DEPC fixation has previously been found to give an excellent preservation of substance P immunoreactivity (3,4). This fixation does not visualize 5-HT.

In order to enable visualization of both 5-HT and substance P immunoreactivity sequentially in one and the same tissue section two alternatives were tried: 1. Non-deparaffinized sections from DEPC-treated material were exposed to formaldehyde vapours for 2 hrs at 80°C. This treatment induced a weak to moderate yellow fluorescence typical of 5-HT in the enterochromaffin cells. 2. Sections from formaldehyde-treated material were used. As could be expected the enterochromaffin cells displayed intense yellow fluorescence due to their content of 5-HT. Several sections were examined and photographed. They were then processed for the immunohistochemical demonstration of substance

P using the PAP technique of Sternberger (10). The substance P antiserum (see ref. 3) (kindly supplied by Dr. Göran Nilsson, Department of Pharmacology, Karolinska Institute, Stockholm, Sweden) was used in dilution 1:20 (alternative 1) or 1:10 (alternative 2). As controls served sections incubated with antiserum inactivated by the addition of excess antigen (100 µg of synthetic substance P per ml diluted antiserum).

Both alternatives permitted the subsequent immunohistochemical demonstration of substance P. However, though clearly visible, the intensity of immunoreaction upon treatment with formaldehyde or DEPC plus formaldehyde was weaker than that seen in sections from material treated with DEPC alone. This could, at least partly, be overcome by using the substance P antiserum in lower dilution. As previously shown substance P immunoreactivity occurred in nerve fibres predominating in the smooth muscle layer and in scattered endocrine-like cells in the mucosal epithelium. Comparison of photographs of 5-HT-fluorescent cells with substance P immunoreactive cells in the same section revealed that the latter cells were identical with 5-HT-containing cells (Fig. 1). From a rough estimate it appears that one out of four enterochromaffin cells in mouse colon displayed substance P immunoreactivity.

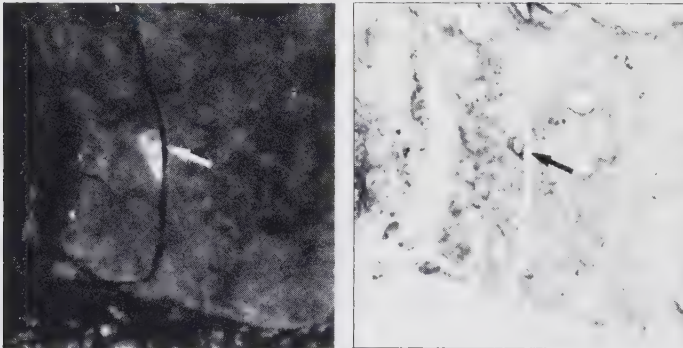


Fig 1.

Mouse colon, formaldehyde vapour fixation. Left: Two adjacent enterochromaffin cells in crypt epithelium displaying intense yellow formaldehyde-induced fluorescence due to their content of 5-HT. Right: Same section processed for immunohistochemical demonstration of substance P (PAP technique). One of the two enterochromaffin cells (arrow) displays substance P immunoreactivity (X 300).

It can thus be concluded that the substance P cells in the gut mucosa are identical with a sub-population of 5-HT-containing enterochromaffin cells. Similar results have recently been obtained by Heitz et al. (11) on rabbit bile duct and human duodenum. However, in contrast to these authors we invariably find substance P immunoreactive cells to contain 5-HT.

Acknowledgement: Grant support from the Swedish Medical Research Council (04X-4499).

References

1. Euler, U.S. von and Gaddum, J.H. *J. Physiol. (Lond.)* 72:74-87, 1931.
2. Chang, M.M., Leeman, S.E. and Niall, H.D. *Nature (Lond.) New Biol.* 232:86-87, 1971.
3. Nilsson, G., Larsson, L.-I., Håkanson, R., Brodin, E., Pernow, B. and Sundler, F. *Histochemistry* 43:97-99, 1975.
4. Pearse, A.G.E. and Polak, J.M. *Histochemistry* 41:373-375, 1975.
5. Håkanson, R. *Acta physiol. scand. Suppl.* 340, 1970.
6. Håkanson, R., Bengmark, S., Brodin, E., Ingemansson, S., Larsson, L.-I., Nilsson, G. and Sundler, F. In: *Proc. Nobel Symp. on Substance P*, New York, Raven Press, 1977.
7. Alumets, J., Håkanson, R., Ingemansson, S. and Sundler, F. *Histochemistry*, in press, 1977.
8. Pearse, A.G.E., Polak, J.M., Adams, C. and Kendall, P.A. *Histochem. J.* 6:347-352, 1974.
9. Björklund, A., Falck, B. and Owman, Ch. In: *Methods of Investigative and Diagnostic Endocrinology*, vol. 1 (J.E. Rall and I.J. Kopin, eds.) p. 318-368, Amsterdam, North-Holland Publ. Comp., 1972.
10. Sternberger, L.A. *Immunocytochemistry*, New Jersey, Prentice-Hall Inc., 1974.
11. Heitz, Ph., Polak, J.M., Timson, C.M. and Pearse, A.G.E. *Histochemistry* 49: 343-347, 1976.

ONTOGENY OF ENDOCRINE CELLS IN PORCINE GUT AND PANCREAS

An immunocytochemical study

J. Alumets, R. Håkanson and F. Sundler

Departments of Histology and Pharmacology, University of Lund, Lund, Sweden

Summary: A number of peptide hormones and hormone candidates were studied by immunocytochemistry with respect to their appearance and development in the developing porcine gastro-entero-pancreatic (GEP) region. The hormones of the pancreatic islets were the first to appear. At 4 weeks of gestation (the earliest stage studied) glucagon, insulin and somatostatin cells occurred in the dorsal pancreatic primordium, whereas PP cells occurred in the ventral primordium. At this time the pancreatic primordia were made up of strands of endocrine cells; no ducts or acini were seen. Subsequently, the endocrine cells were dispersed by the growing exocrine parenchyma and at still later stages they aggregated in small nests. Not until around birth did they form typical mantle islets of the adult type. In the gut, glucagon cells were first to appear. They were observed in the upper small intestine at the 4 week stage; later they disappeared from this location but appeared instead in the lower small intestine and colon where they remained. Gastrin and somatostatin cells appeared earlier in the stomach (at the 5 week stage) than in the intestines (at the 6 week stage). Gastrin cells were not seen in the pancreas during any developmental stage. Secretin, CCK, motilin, GIP and neurotensin cells all appeared at the 6-8 week stage and were restricted to the small intestine throughout development. Enkephalin immunoreactive cells appeared late during ontogeny (at the 13-15 week stage) in both the gut and pancreas. In view of the early appearance of many GEP endocrine cell types in fetal life, a functional significance of GEP hormones in early development is suggested.

The gut and pancreas harbour an impressive number of peptide hormones and hormone candidates¹. The generation of specific antisera towards many of them has led to the localization of their cellular storage sites by means of immunocytochemistry. Thus, data are available on the occurrence and distribution of more than fifteen peptide hormone-producing cell types in the gut and pancreas of a variety of species². However, information on their ontogeny is limited to a few peptides and to a few species only, most of them common laboratory animals, the rat in particular³⁻¹⁸. Information on larger mammals is scarce presumably due to difficulties in obtaining fetal material covering all developmental stages. Thus, most studies in man^{9,15,19-35} have concentrated on a short period at mid gestation and immediately after parturition. However, pig fetuses from all developmental stages are accessible due to the high frequency of incidental pregnancies discovered at slaughter. The present report gives a detailed account of the development of several gastro-entero-pancreatic (GEP) endocrine cell types in fetal and neonatal pigs.

MATERIAL AND METHODS

Tissue material from fetal and adult pigs was obtained from a local slaughter house and newborn and young piglets from a local breeder. Fetuses were obtained from pigs found to be pregnant at the time of slaughter. Piglets were killed by an overdose of sodium pentobarbitone. Tissue specimens were collected within 10 minutes after death from different parts of the gastrointestinal tract and from the pancreas of fetuses (1.5 - 28 cm crown-rump length, i.e. 4 - 16 weeks of gestational age), new-born (0 - 17 days old) and adult pigs. Whenever possible, specimens were taken from both the duodenal and splenic portions of the pancreas. The relation between gestational age and crown-rump length in pig fetuses has been summarized by Book and Bustad³⁶. In the present study the crown-rump length was transformed to fetal age from the data presented by these authors. In the following the stages are given as weeks of gestation.

The specimens were freeze-dried and vapour fixed with diethyl pyrocarbonate³⁷ (3 hrs, 55°C) and embedded in paraffin in vacuo. The indirect immunofluorescence method³⁸ or the peroxidase-antiperoxidase (PAP) technique³⁹ were used for the immunocytochemical demonstration of a variety of peptide hormones and hormone

candidates. Sections, 6 μm in thickness, were deparaffinized in xylene and hydrated in graded ethanol solutions and exposed to peptide antiserum for 3 h at room temperature (immunofluorescence) or for 24 h at 4°C (PAP technique). After rinsing in phosphate buffer, a second layer of fluorescein-labelled or unlabelled goat anti-rabbit IgG (dilution 1:20) was applied for 30 minutes at room temperature. In the PAP procedure this was followed by incubation for 1 h with PAP complex (dilution 1:320), and the site of antigen-antibody reaction was revealed by incubation with a solution containing diaminobenzidine and hydrogen peroxide³⁹. Details on the various peptide antisera are given in Table 1. All antisera have been used in previous immunocytochemical studies and most of the antisera have been characterized in detail elsewhere (see Table 1). Control sections were incubated with antisera inactivated by the addition of the respective antigen in excess (10-100 μg of synthetic or pure antigen per ml diluted antiserum).

Fluorescein labelled- or unlabelled goat anti-rabbit IgG was purchased from SBL, Stockholm, Sweden and PAP complex was obtained from Dako, Copenhagen, Denmark.

RESULTS

The time and sites of appearance of various GEP hormone producing endocrine cells are illustrated in Figs. 1-4.

Insulin

Immunoreactive cells were found in the pancreas only. They were first observed as an accumulation of cells situated near the dorsal side of the gut wall at a fetal age of about 4 weeks (the earliest stage studied) (Fig. 5a and 6a). At this stage the dorsal pancreatic primordium was made up of strands of epithelial cells of which the insulin cells constituted a significant proportion. At the 10 week stage insulin cells appeared in the duodenal portion of the pancreas, which is a derivative of the ventral pancreatic bud. The cells were few and weakly immunoreactive at this stage. Subsequently, the number of insulin cells increased rapidly, both in the duodenal and splenic portion of the pancreas. At the 13 week stage the insulin cells were randomly dispersed, separated by the growing exocrine parenchyma (Fig. 7). Not until about 10-13 days after birth did the cells cluster together in small islets. Still, rather many cells remained scattered in the exocrine parenchyma. In the adult pig, however, extra-insular cells were very few.

The antiserum used demonstrates both pancreatic-type and gut-type glucagon.

Fairly numerous immunoreactive cells were first found in the dorsal pancreatic primordium close to the gut of about 4 weeks old fetuses (the earliest stage studied) (Fig. 5b, 6b and 8). At first they were densely packed but subsequently (after 2 - 4 weeks), like the insulin cells, they occurred dispersed in the parenchyma; only few small islet-like nests were seen (Fig. 9). A few weeks later the islets were larger and more numerous. Glucagon and insulin cells were randomly distributed within the islets. In the duodenal portion, derived from the ventral primordium, glucagon cells appeared late (the 10 week stage) and remained relatively few during development. At parturition the glucagon cells predominated in the islets but many cells remained scattered in the exocrine parenchyma. Two weeks after birth the adult distribution pattern was established: glucagon cells were found in mantle islets where they predominated in the islet periphery with insulin cells occupying the central core.

In the upper small intestine glucagon immunoreactive cells were first seen in 4 week old fetuses. At this stage they were very few. Most of them were situated basally in the stratified epithelium (Fig. 8 and 10), which at this stage obliterates the lumen of the gut (Fig. 10). The immunoreactive cells occurred in the gut segment adjacent to the pancreatic primordium. The number of immunoreactive cells in the epithelium of the most proximal part of the small intestine at first increased slowly but then decreased; the cells had disappeared altogether from this location at a fetal age of 13 weeks. In the distal small intestine and large intestine glucagon immunoreactive cells appeared at the 8 week stage; here they increased progressively in number up to the 13 week stage. Glucagon cells were numerous in this location from 14 - 15 weeks on. In the body of the stomach glucagon immunoreactive cells first appeared basally in the glands at the 9 week stage; at 14 weeks the cells were still few and scattered; they remained so during the subsequent stages. Very few glucagon cells were found in the antrum between 8 and 13 weeks; they were not found here either before or after.

Somatostatin

Immunoreactive cells were seen in the dorsal pancreatic primordium at a gestational age of about 4 weeks; they occurred intermingled with glucagon and insulin cells. The somatostatin cells were scattered and fewer in number than the insulin and glucagon cells at the corresponding stage (Fig. 5c). Within the next few weeks the number of somatostatin cells increased greatly. At about a fetal age of 10 weeks most somatostatin cells occurred in small nests; a few remained scattered in the exocrine parenchyma (Fig. 11). No difference between the duodenal and splenic parts of the pancreas could be found at this time. A few weeks after birth, mantle islets of the adult type were composed of somatostatin cells and glucagon cells surrounding the insulin cells.

In the stomach the first somatostatin immunoreactive cells were found in 4 - 5 weeks old fetuses (Fig. 12). At this stage they were very few. At about the 6 - 7 week stage the somatostatin cells were still few but regularly found. One week later they were observed also in the duodenum, and another 1 - 2 weeks later they occurred in the rest of the small intestine. In the large intestine the first somatostatin cells were found at the fetal age of 7 weeks. At this stage the intestinal somatostatin cells were very few and situated basally in the epithelium. Subsequently, they increased slowly in number; they were regularly found at the 12 - 13 week stage. Not until at 15 - 16 weeks did somatostatin cells appear in the oxyntic gland area of the stomach. At birth the somatostatin cells were rather frequent all along the gut rather reminiscent of the situation in the adult pig.

Pancreatic polypeptide (PP)

The ventral pancreatic primordium contained PP immunoreactive cells at the 4 week stage (the earliest stage studied) (Fig. 13). The ventral pancreatic bud differed from the dorsal bud in that no glucagon, insulin or somatostatin cells were observed at this stage. The PP cells were disseminated in the growing exocrine parenchyma during the subsequent 2-4 weeks. Their number and the intensity of their immunostaining increased rapidly. From a fetal age of 13 weeks until parturition, the duodenal portion of the pancreas was rich in PP cells, some of which were arranged in clusters, while others occurred scattered in the exocrine parenchyma. After parturition the PP cells diminished in frequency. At 12-14 days after birth the cells were

still fairly numerous, occurring as single cells in the exocrine parenchyma or in small clusters within or outside the islets. The dorsal pancreatic bud lacked PP cells and they were not found in the corresponding splenic portion of the pancreas until at the 13 week stage. They remained few in this location throughout development.

Immunoreactive PP cells were first seen in the duodenum at a fetal age of 8 weeks and they were regularly seen at the 10 week stage; they increased slowly in number during the following 2 - 3 weeks. Their number did not change during subsequent stages. PP cells were seen in the antrum from the 6 week stage (Fig. 14) until birth and in the oxyntic gland area of 2 week old piglets. PP immunoreactive cells were not found in the jejunum-ileum or in the colon at any stage.

Secretin

Immunoreactive secretin cells were first seen in the duodenum at a fetal age of 6 weeks (Fig. 15). Their number increased gradually up to parturition when they reached the adult frequency of occurrence; they predominated on the villi and in the transitional zone between crypts and villi. Immunoreactive cells were seen in the upper jejunum at the 8-9 week stage. They were rare in this location until a fetal age of 14 weeks. The cells then increased gradually in number until birth. They remained fairly few postnatally in this location. They were very few before and after parturition in the remainder of the jejunum. In no other part of the gut nor in the pancreas were secretin immunoreactive cells detected.

Gastrin/CCK

One of the gastrin antisera used (code no 4562) is directed against the COOH-terminal four amino acids of the gastrin molecule, a sequence common to gastrin and CCK⁷. Thus, this antiserum stains not only gastrin cells but also CCK cells as well as other cells containing the COOH-terminal tetrapeptide of gastrin/CCK. Cells containing the COOH-terminal part of the gastrin molecule (gastrin/CCK cells) were first observed immunocytochemically in the lower stomach, probably corresponding to the antro-pyloric region, at a gestational age of 4-5 weeks (Fig. 2 and 16a). At this stage the gastrin/CCK immunoreactive cells were few in number and located basally in the stratified epithelium. One week later they were seen also in the duodenum (Fig. 16b). From the 7th week on gastrin/CCK

immunoreactive cells were numerous in the antrum; they were fewer in the duodenum. At the 8-9 week stage gastrin/CCK cells appeared in the remainder of the small intestine. Some weeks later gastrin/CCK cells were seen in the large intestine; they were few in number and disappeared at a fetal age of approximately 15 weeks. No gastrin/CCK immunoreactive cells were detected in the pancreas at any stage.

Gastrin

Antiserum no L6 is specific for gastrin-17 and does not cross-react with CCK⁴⁰. Gastrin-immunoreactive cells were observed in the lower stomach at a gestational age of 4-5 weeks; one week later they appeared in the duodenum (Fig. 2). From the 8th week on, gastrin cells were regularly found in both antrum and duodenum. In the antrum their number increased gradually; the adult frequency was reached at a fetal age of 15-16 weeks. In the proximal duodenum gastrin cells were fairly numerous from the 8th week on. It should be noted that the gastrin cells are included among those referred to as gastrin/CCK cells.

CCK

The CCK antiserum is thought to demonstrate CCK cells specifically.

CCK immunoreactive cells appeared in the duodenum at a fetal age of 7 weeks (Fig. 2 and 17). Their number increased gradually to reach the adult frequency of occurrence at about the 13 week stage, when they were fairly numerous and situated mainly in the basal parts of the crypts. Also in the rest of the small intestine were immunoreactive cells regularly seen but their number was low. Immunoreactive CCK cells appeared in the lower part of the stomach (probably antrum) at the 7 week stage; the number of cells increased slowly during the 4-5 weeks that followed but later their number diminished and at term they had disappeared altogether. No CCK cells could be detected in the oxyntic gland area, pancreas or colon. Also the CCK cells are included among those referred to as gastrin/CCK cells.

Motilin

Immunoreactive cells appeared first in the duodenum at a fetal age of 9 weeks. They had reached the adult frequency of occurrence already at the 13 week stage. They predominated in the crypts; only rarely did they occur on the villi. In the

jejunum the first motilin cells were seen in 14 week old fetuses; the cells remained few in this location during subsequent stages. Motilin cells were not detected in other parts of the gut nor in the pancreas.

Neurotensin

Immunoreactive cells were found in the small intestine only, with a predominance in the ileum and distal jejunum; in the duodenum they were very few. They were first detected in the ileum at a fetal age of 8 weeks. Their number increased slowly up to parturition when the adult frequency was established. They occurred mainly on the villi.

Gastric Inhibitory Peptide (GIP)

Two GIP antisera with different specificity were used. One antiserum (R-65) demonstrated cells in the upper small intestine exclusively⁴¹. The other antiserum (RD 11/10/77) in addition demonstrated cells in the pancreas and the rest of the gut; these latter cells were identical with the glucagon immunoreactive cells. This antiserum therefore probably demonstrates not only GIP but also GIP-like peptides, similar to but distinct from authentic GIP^{41,42}. None of the antisera cross-reacts with glucagon.

Using antiserum R-65 immunoreactive cells appeared in the most proximal part of the small intestine (duodenum) at about the 6-7 week stage (Fig. 18a). The cells increased slowly in number during the subsequent weeks, but they remained rather few until birth (Fig. 18b). After parturition they were moderate in number and had a distribution as in the adult pig. About 2 weeks after their appearance in the duodenum the cells appeared also in the remainder of the small intestine, but here they were few throughout development, as in the adult pig.

The second GIP antiserum (RD 11/19/77) in addition demonstrated the glucagon immunoreactive cells in the stomach, lower intestine and pancreas. The pancreatic glucagon cells contained GIP immunoreactive material already when they first appeared (i.e. at 4 weeks gestation). The GIP immunoreactive material displayed the same distribution and developmental pattern as did glucagon cells.

Enkephalin

Immunoreactive epithelial cells were first observed in the duodenum where they occurred basally in the epithelium at a fetal age of 13 weeks (Fig. 19a). Their number

increased rapidly up to parturition. In the antrum they were first seen a few days after birth. Two weeks later they had reached the adult frequency of occurrence in the antrum and proximal small intestine. In the pancreas scattered immunoreactive cells were seen immediately before birth. They predominated in the duodenal part. At birth and later they were regularly found scattered in the exocrine parenchyma (Fig. 19b). They were never seen in the islets.

"ACTH"

Cells reacting with antisera* raised against pure porcine ACTH were found throughout the gut and pancreas. The first immunoreactive cells were detected in the pancreas in 10 week-old fetuses and found to be randomly distributed in the parenchyma. At this stage they were few in number; they became gradually more numerous until term (Fig. 20). In the distal small intestine few weakly immunoreactive cells appeared at the 10 week stage; they remained few during subsequent stages. At the 13 week stage immunoreactive cells appeared also in the colon where they became rather numerous within the next 3 to 5 weeks. One week after birth the cells in the small and large intestines and in the pancreas decreased gradually in number; in the adult animal they were few and only weakly immunoreactive. In the pancreas the cells were scattered in the exocrine parenchyma. Immunoreactive cells appeared in the oxyntic gland area and antrum at birth, but they remained few and stained only weakly.

Substance P

No substance P immunoreactive cells were observed at any developmental stage. They were absent also in the adult pig.

*Footnote: It should be noted that the immunoreactive material is unrelated to ACTH but instead reflects the presence of a population of antibodies in the ACTH antisera directed against a contaminant in the ACTH preparation used for immunization. The identity of this contaminant, which probably stems from the ACTH/MSH cells, is unknown. The cells in the pancreas and gut that stain with the ACTH antiserum have previously been shown to be distinct from other known endocrine cells⁴³.

DISCUSSION

Several precious reports have dealt with the ontogeny of peptide hormone-producing cells in the mammalian gut and pancreas. Most of them describe the development of the pancreatic islet cells, notable the insulin cells^{3,4,8,10,16,18,23} and glucagon cells^{3,5,8,10,16,18,20,23}, but also the somatostatin cells^{8-10,16,18,22,26,33,34} and the PP cells^{10,13,16,18,30}; most of these studies concern the rat (for refs. see introduction). This is because the rat is a widely used laboratory animal, and because the development can be followed throughout fetal and neonatal life. The rat differs, however, from higher mammals in that the period of fetal development is short and that the neonate is immature. This discrepancy makes it difficult to compare the ontogeny of the rat with that of higher species. The pig, however, is thought to be fairly similar to man in its fetal development³⁶ and may be representative of higher mammals.

The results of the present study indicate that of the various GEP endocrine cells those in the pancreas are among the first to appear; they were found at the earliest stage studied, i.e. at the 4 week stage¹⁶. Glucagon, insulin and somatostatin cells, in decreasing order of frequency, are all found in the dorsal pancreatic primordium, which seems to be wholly made up of densely packed endocrine cells^{44,45}. PP cells, however, are first found in the ventral pancreatic primordium^{13,30,32,45}. At this early stage no ducts or acini are seen in the pancreatic primordia. During subsequent stages the individual endocrine cells are separated from each other, probably as a result of the growth of the exocrine parenchyma. Later on the pancreatic endocrine cells form nests and finally mantle islets of the adult type. Postnatally some insulin, glucagon and somatostatin cells remain scattered in the exocrine parenchyma. The adult distribution pattern is not reached until several months after birth. The PP cells appear late and are few in the splenic part which originates from the dorsal bud^{13,30,32,44,45}. This is in contrast to the glucagon cells, which appear late and remain scarce in the duodenal lobe^{44,45}.

The gastrin/CCK family of peptides was studied using three antisera with different region specificity; one demonstrating gastrin-17 specifically, another CCK, and the third the COOH-terminal tetrapeptide common to gastrin and CCK. The present results suggest that the latter antiserum detects not only gastrin cells and CCK cells but also a third cell population⁵⁶ with unidentified peptide hormones and unknown physiological

function. Gastrin cells appear first in the antrum (4-5 week stage) and the CCK cells first in the duodenum (6-7 week stage). The third cell population included among those demonstrated by the non-specific antiserum appeared gradually in the small and large intestines.

Larsson and Rehfeld⁵⁶ have presented evidence that the gastrin COOH-terminal tetrapeptide (tetragastrin) is the quantitatively predominant form of gastrin in the porcine digestive tract and that tetragastrin occurs in a special endocrine cell type, referred to as the tetragastrin cell (TG cell). This cell type occurs predominantly in the intestines and is only rarely found in the antrum. This conclusion is at odds with the findings of Rehfeld and Larsson⁵⁷, who claim that the antrum is the richest source of tetragastrin.

It is notable that most endocrine cells in the digestive tract, except those producing somatostatin, gastrin and neurotensin, appear first in the most proximal part of the small intestine. From this foothold they "invade" other regions of the gut, gradually appearing proximally and/or distally to this point. Similar observations have been made in man^{25,34} and rat⁶. The somatostatin and gastrin cells appear in the antrum before they appear in the duodenum. The situation is different in man^{25,33} and rat^{6,7} where gastrin immunoreactive cells seem to occur earlier in the duodenum than in the antrum. No gastrin or CCK cells could be demonstrated in the pancreas at any stage. This is notable since such cells have previously been demonstrated in the fetal and neonatal rat pancreas^{6,7,16}.

CCK cells appear in the antrum soon after the appearance of gastrin cells; at about the same time CCK cells are found in the duodenum. The antral CCK cells, after an initial slow increase in number, disappear before birth^{10,15,27,35}.

The glucagon immunoreactive cell is another example of a cell type that occurs transiently during development in a certain location. Glucagon immunoreactive cells appear first in the duodenum¹⁶, only to disappear from this location during subsequent stages. In the remainder of the small intestines, in the large intestines and in the oxyntic gland area the glucagon immunoreactive cells appear later than in the duodenum, but in these locations the cells remain permanently^{21,22,31}.

The duodenal glucagon cells are the first endocrine cells to be found in the gut. They were present already at the earliest stage studied, i.e. at the 4th week of gesta-

tion, situated in the gut segment adjacent to the pancreatic primordium¹⁶. At this time the intestine forms a narrow tube; the lumen is almost completely obliterated by a stratified epithelium.

The early appearance of glucagon cells in the duodenum and pancreas of the rat and man have been described previously. The development of pancreatic glucagon cells, and insulin cells has been extensively studied in these two species with conventional staining techniques and with immunocytochemical methods. It is notable that the latter methods seem capable of detecting these cells earlier than do other histological and histochemical methods. Thus, insulin cells in the pancreas of fetal rat have been detected at an earlier stage with immunocytochemical techniques than with e.g. the aldehyde fuchsin method^{4,8,16}. Similarly, the glucagon and somatostatin cells can be demonstrated earlier with immunocytochemical methods than with other staining techniques⁸. Conceivably, most of these staining techniques demonstrate granule-stored material which is associated with or related to the peptide hormone(s). The lack of developmental parallelism, however, may indicate that various granule constituents of endocrine cells develop according to different time scales and that the cells may not be fully mature despite the presence of demonstrable peptide hormone. For instance, bioactive insulin is detected much later than immunoreactive insulin⁴. Also the varying proportions of different molecular forms of gastrin during ontogeny supports this view³³.

The secretin, neurotensin and motilin cells are restricted to one and the same gut segment throughout development. Thus, secretin^{10,11,15,22,35} and motilin³⁵ cells are found in the small intestine only, with the earliest appearance in the duodenum and proximal jejunum where they predominate. Throughout development neurotensin cells are more numerous in the jejunum-ileum than in the duodenum, where only few scattered cells are found^{12,28}.

GIP was originally isolated from the upper small intestine and later localized to endocrine cells in the duodenum⁴⁶. Certain GIP antisera demonstrate cells restricted to the small intestine during all stages of development^{17,41,46}. Other GIP antisera, however, in addition demonstrate glucagon cells both in the pancreas and gut^{41,42}. In these latter cells the GIP-like material, which is similar to but probably not identical with authentic GIP, appears at the same time as glucagon.

Enkephalin immunoreactive cells are 5-hydroxytryptamine-containing, and are

found in the gut in large numbers only in the pig⁴⁷. They appear late in comparison with other endocrine cell types. They are seen first in the duodenum a few weeks before parturition, and appear in the antrum and pancreas at birth.

It has become increasingly evident that the fetal development of the many diverse GEP endocrine cell types is carefully co-ordinated in time and space⁴⁸. Certain gut hormones may exert trophic or antitrophic effects on the gut and pancreas^{49,50} and others may initiate gastrointestinal secretion and motility during ontogeny^{51,52}. Such effects probably result from co-operation between several peptide hormones. The time-table for the appearance of the various gut hormones is therefore of interest. Knowledge of their occurrence and distribution in the gut and pancreas during development will assist in defining their functional roles during ontogeny.

Acknowledgements: This work was supported by grants from the Swedish Medical Research Council (Project No 04X 4499) and the Pahlsson's Foundation.

REFERENCES

1. Glass GBJ (Ed.): *Gastrointestinal Hormones*, New York, Raven Press, 1980
2. Solcia E, Polak JM, Pearse AGE, et al: Lausanne 1977 classification of gastroenteropancreatic endocrine cells. In: *Gut Hormones*. Ed by SR Bloom, Churchill-Livingston, 1978, pp 40-48
3. Hard WL: The origin and differentiation of the alpha and beta cells in the pancreatic islets of the rat. *Am J Anat* 75: 369-394, 1944
4. Grillo TAI: The occurrence of insulin in the pancreas of foetuses of some rodents. *J Endocrin* 31: 67-73, 1964
5. Orci L, Lambert AE, Rouiller CH, et al: Evidence for the presence of A-cells in the endocrine fetal pancreas of the rat. *Horm Metab Res* 1: 108-110, 1969
6. Larsson L-I, Håkanson R, Rehfeld JF, et al: Occurrence and neonatal development of gastrin immunoreactivity in the digestive tract of the rat. *Cell Tiss Res* 149: 275-281, 1974
7. Larsson, L-I, Rehfeld JF, Sundler F, et al: Pancreatic gastrin in foetal and neonatal rats. *Nature* 262: 609-610, 1976
8. Schweisthal MR, Frost CC, Brinn JE: Ontogeny of four cell types in fetal rat islets using histochemical techniques. *Acta diabet lat* 13: 30-39, 1976
9. Alumets J, Sundler F, Håkanson R: Distribution, ontogeny and ultrastructure of somatostatin immunoreactive cells in the pancreas and gut. *Cell Tiss Res* 185: 465-479, 1977
10. Larsson, L-I: Ontogeny of peptide-producing nerves and endocrine cells of the gastro-duodeno-pancreatic region. *Histochemistry* 54: 133-142, 1977
11. Larsson L-I, Sundler F, Alumets J, et al: Distribution, ontogeny and ultrastructure of the mammalian secretin cell. *Cell Tiss Res* 181: 361-368, 1977.
12. Sundler F, Håkanson R, Hammer RA, et al: Immunohistochemical localization of neurotensin in endocrine cells of the gut. *Cell Tiss Res* 178: 313-321, 1977
13. Sundler F, Håkanson R, Larsson L-I: Ontogeny of rat pancreatic polypeptide (PP) cells. *Cell Tiss Res* 178: 303-306, 1977
14. Larsson L-I: ACTH-like immunoreactivity in the gastrin cell. Independent changes in gastrin and ACTH-like immunoreactivity during ontogeny. *Histochemistry* 56: 245-251, 1978

15. Larsson, L-I, Jørgensen LM: Ultrastructural and cytochemical studies on the cyto-differentiation of duodenal endocrine cells. *Cell Tiss Res* 194: 79-102, 1978
16. Fujii S: Development of pancreatic endocrine cells in the rat fetus. *Arch histol jap* 42: 467-479, 1979
17. Gespach C, Bataille D, Jarrousse C, et al: Ontogeny and distribution of immunoreactive gastric inhibitory polypeptide (IR-GIP) in rat small intestine. *Acta endocr (Kbh)* 90: 307-316, 1979
18. McEvoy RC, Madson KL: Pancreatic insulin-, glucagon-, and somatostatin-positive islet cell populations during the perinatal development of the rat. I Morphometric quantitation. *Biol Neonate* 38: 248-254, 1980
19. Adesanya T, Grillo I, Shima K: Insulin content and enzyme histochemistry of the human foetal pancreatic islet. *J Endocrin* 36: 151-158, 1966
20. Björkman N, Hellerström C, Hellman B, et al: The cell types in the endocrine pancreas of the human fetus. *Z Zellforsch* 72: 425-445, 1966
21. Assan R, Boillot J: Pancreatic glucagon, and glucagon-like material in tissues and plasmas from human fetuses 6-26 weeks old. *Metabolic Processes in the Foetus and Newborn Infant*. Ed by Jonxis et al, 1971, pp 210-219.
22. Polak JM, Coulling I, Bloom S, et al: Immunofluorescent localization of Secretin and Enteroglucagon in Human Intestinal Mucosa. *Scand J Gastroent* 6: 739-744, 1971.
23. Like AA, Orci L: Embryogenesis of the human pancreatic islets: a light and electron microscopic study. *Diabetes* 21 (Suppl 2): 511-534, 1972
24. Dubois PM, Paulin C, Assan R: Evidence for immunoreactive somatostatin in the endocrine cells of human foetal pancreas. *Nature* 256: 731-732, 1975.
25. Larsson L-I, Håkanson R, Sjöberg N-O, Sundler F: Fluorescence histochemistry of the gastrin cell in fetal and adult man. *Gastroenterology* 68: 1152-1159, 1975
26. Dubois PM, Paulin C, Chayvialle JA: Identification of gastrin-secreting cells and cholecystokinin-secreting cells in the gastrointestinal tract of the human fetus and adult man. *Cell Tiss Res* 175: 351-356, 1976
27. Dubois PM, Paulin C: Gastrintestinal somatostatin cells in the human fetus. *Cell Tiss Res* 166: 179-184, 1976
28. Helmstaedter G, Mühlmann G, Feurle GE, et al: Immunohistochemical identification of gastrointestinal neurotensin cells in human embryos. *Cell Tiss Res* 184: 315-320, 1977

29. Larsson L-I, Rehfeld JF, Goltermann N: Gastrin in the human fetus. Distribution and molecular forms of gastrin in the antro-pyloric gland area. Duodenum and pancreas. *Scand J Gastroent* 12: 869-872, 1977
30. Paulin C, Dubois PM: Immunohistochemical identification and localization of pancreatic polypeptide cells in the pancreas and gastrointestinal tract of the human fetus and adult man. *Cell Tiss Res* 188: 251-257, 1978
31. Lehy T, Cristina ML: Ontogeny and distribution of certain endocrine cells in the human fetal large intestine. *Cell Tiss Res* 203: 415-426, 1979
32. Rahier J, Wallon J, Gepts W, et al: Localization of pancreatic polypeptide cells in a limited lobe of the human neonate pancreas: remnant of the ventral primordium? *Cell Tiss Res* 200: 359-366, 1979
33. Track NS, Creutzfeldt C, Litzenberger J, et al: Appearance of gastrin and somatostatin in the human fetal stomach, duodenum and pancreas. *Digestion* 19: 292-306, 1979
34. Chayvialle JA, Paulin C, Dubois PM, et al: Ontogeny of somatostatin in the human gastro-intestinal tract, endocrine pancreas and hypothalamus. *Acta Endocr (Kbh)* 94: 1-10, 1980
35. Iwanaga T, Kobayashi S, Fujita T, et al: Immunocytochemistry and ultrastructure of Segi's cap. *Biomed Res* 1: 117-129, 1980
36. Book SA, Bustad LK: The fetal and neonatal pig in biomedical research. *J Anim Sci* 38: 997-1002, 1974
37. Pearse AGE, Polak JM: Bifunctional reagents as vapour- and liquid-phase fixatives for immunohistochemistry. *Histochem. J.* 7: 179-186, 1975
38. Coons AH, Leduc EH, Connolly JM: Studies on antibody production. I A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J Exp Med* 102: 49-60, 1955
39. Sternberger LA: Immunocytochemistry. New Jersey: Prentice Hall Inc. 1974
40. Vaillant C, Dockray G, Hopkins CR: Cellular origins of different forms of gastrin. The specific immunocytochemical localization of related peptides. *J Histochem Cytochem* 25: 932-935, 1979
41. Sjölund K, Ekelund M, Alumets J, et al: Immunoreactive GIP in glucagon cells: Properties and origin. An immunocytochemical study using several GIP antisera. In manuscript

42. Alumets J, Håkanson R, O'Dorisio T, et al: Is GIP a glucagon cell constituent? *Histochemistry* 58: 253-257, 1978
43. Sundler F, Alumets J, Ekman R, et al: Immunoreactive ACTH in Porcine Gut and Pancreas: Fact or Artifact? *J Histochem Cytochem*, in press, 1981
44. Bencosme SA, Liepa E: Regional differences of the pancreatic islet. *Endocrinology* 57: 588-593, 1955
45. Baetens D, Malaisse-Lagae F, Perrelet A, et al: Endocrine pancreas: Three-dimensional reconstruction shows two types of islet of Langerhans. *Science* 206: 1323-1325, 1979
46. Polak JM, Bloom SR, Kuzio M, et al: Cellular localization of gastric inhibitory polypeptide in the duodenum and jejunum. *Gut* 14: 284-288, 1973
47. Alumets J, Håkanson R, Sundler F, et al.: Leu-Enkephalin-like material in nerves and enterochromaffin cells in the gut. *Histochemistry* 56: 187-196, 1978
48. Snow MHL, Tam PPL: Timing in embryological development. *Nature* 286: 107, 1980
49. Johnson LR: Regulation of gastrointestinal mucosal growth. *World J Surg* 3: 477-487, 1979
50. Oscarson J, Håkanson R, Liedberg G, et al: Variated serum gastrin concentration: Trophic effects on the gastrointestinal tract of the rat. *Acta Physiol scand*, Suppl. 475, 1979
51. Enochs MR, Johnson LR: Hormonal regulation of gastrointestinal tract growth; biochemical and physiologic aspects. *Progress in Gastroenter* 3: 3-28, 1977
52. Grasso S, Fallucca F, Mazzone D, et al: Insulin and glucagon secretion in the human fetus and newborn. In: *Proc. of the Sero symposia*, Vol. 30. Ed by D Andreani et al. Academic Press, London, 1980, pp. 83-91
53. Heding LG: Determination of total serum insulin (IRI) in insulin treated diabetic patients. *Diabetologia* 8: 260-266, 1972
54. Schlegel W, Raptis S: A reliable method for generating antibodies against pancreaticozym, secretin and gastrin. *Clinica Chemica Acta* 73: 439-444, 1977
55. Yanaihara N, Yanaihara C, Nagai K, et al: Motilin-like immunoreactivity in porcine, canine, human and rat tissues. *Biomedical Research* 1: 76-83, 1980

56. Larsson LI, Rehfeld JF: A peptide resembling COOH-terminal tetrapeptide amide of gastrin from a new gastrointestinal endocrine cell type. *Nature* 277: 575-578, 1979
57. Rehfeld JF, Larsson LI: The predominating molecular form of gastrin and cholecystokinin in the gut is a small peptide corresponding to their COOH-terminal tetrapeptide amide. *Acta physiol. scand.* 105: 117-119, 1979

TABLE 1

Source and working dilutions of the antisera used

Antisera against	Antigen	Immu- fluorescence	Working dilution PAP staining	Code	Source	Refs.
Insulin	Pure bovine insulin	1:80	1:5120	LAI	L. Heding, Novo Res. Inst., Copenhagen, Denmark	53
Glucagon	Pure porcine pancreatic glucagon	1:160	1:640	7811 ¹⁾	Milab, Malmö, Sweden	41
Somatostatin	Synthetic ovine somatostatin	1:40	1:1280	19576 19578	M.P. Dubois, Inst. Nat. Rech. Agr., Nouzilly, France	9
Pancreatic Polypeptide (PP)	Pure bovine PP	1:80	1:2560	BPP	R. Chance, Eli Lilly, Indianapolis, Ind., USA	13
Secretin	Pure porcine secretin	1:80	1:20000	5585	O.B. Schaffalitzky de Muckadell and J. Fahrenkrug, Dept. of Clin.Chem., Bispebjerg Hospital, Copenhagen, Denmark	11
Gastrin	1) Synthetic human gastrin 2-17 2) Synthetic human gastrin 1-17	1:640 —	1:10240 1:600	4562 ²⁾ L6 ³⁾	J.F. Rehfeld, Dept. Med. Chem., Århus Univ., Århus, Denmark G. Dockray, Dept. of Physiology, Univ. of Liverpool, England	7 40
CCK	Pure porcine CCK-33	1:10	1:320	4)	W. Schlegel, Dept. of Gynecology, Univ. of Münster, Germany	54
Motilin	Synthetic motilin	1:80	1:5120	GP 1103	N. Yanaihara, Dept. of Bioorganic Chemistry, College of Pharmacy, Schizuoka, Japan	55
Neurotensin	Synthetic neurotensin	1:40	1:640	HC-8	R. Carraway, Dept. of Phys. and Lab. of Human Reproduction, Harvard Med. School, Boston, Mass., USA	12

GIP	1) Pure porcine GIP	1:80	1:10240	RD 11/10/77 ⁵⁾	T. O'Dorisio, Div. of Endocrine and Metabolism, Ohio State Univ., Columbus, Ohio, USA	42
	2) Pure porcine GIP	—	1:240	R-65 ⁶⁾	A. J. Moody, Novo Res. Inst., Copenhagen, Denmark	41
Enkephalin	Synthetic leu-enkephalin	1:60	1:240	"leu-enk" ⁷⁾	R. J. Miller and K.-J. Chang, Wellcome Res. Lab., Burroughs Wellcome Co., Triangle Park, N.C., USA	47
ACTH	Pure porcine ACTH 1-39	1:80	1:640	No. 1	Own	43
Substance P	Synthetic bovine substance P	1:80	1:320	SP 8	P. C. Emson, Med. Res. Council, Cambridge, England	

Comments on the antisera used

- 1) This antiserum cross-reacts with gut-type glucagon (glicentin).
- 2) This antiserum is directed against the COOH-terminal four amino acids, common to gastrin and CCK. It therefore cross-reacts with CCK at the immunocytochemical level.
- 3) This antiserum is specific for gastrin-17 and does not cross-react with CCK.
- 4) This antiserum does not cross-react with gastrin.
- 5) Besides GIP cells this antiserum stains the glucagon cells in the pancreas and the glicentin cells in the gut.
- 6) This antiserum stains GIP cells in the duodenum but not glucagon cells in the pancreas or glicentin cells in the gut.
- 7) This antiserum cross-reacts with met-enkephalin at the immunocytochemical level.

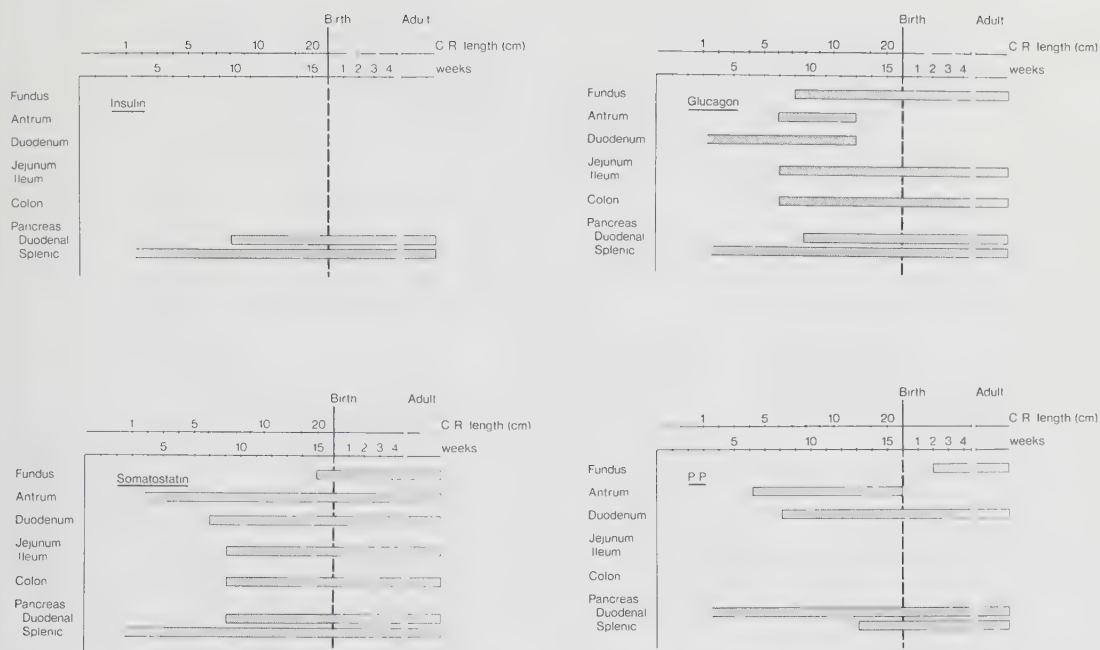


Fig. 1. Time and sites of appearance of cells storing insulin, glucagon, somatostatin and pancreatic polypeptide (PP) in pancreas and gut of the fetal and newborn pig.

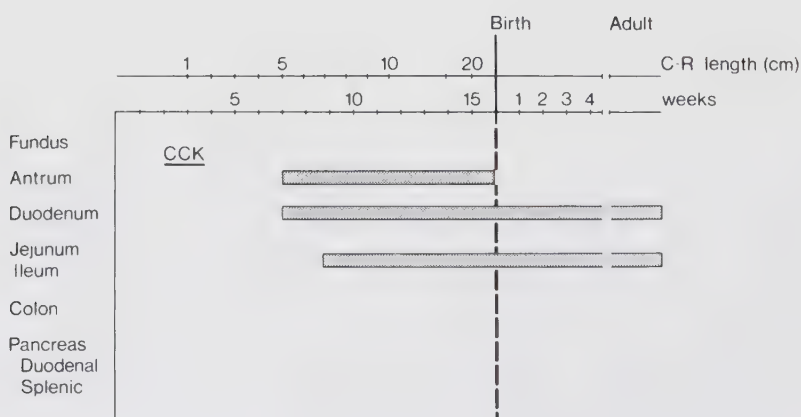
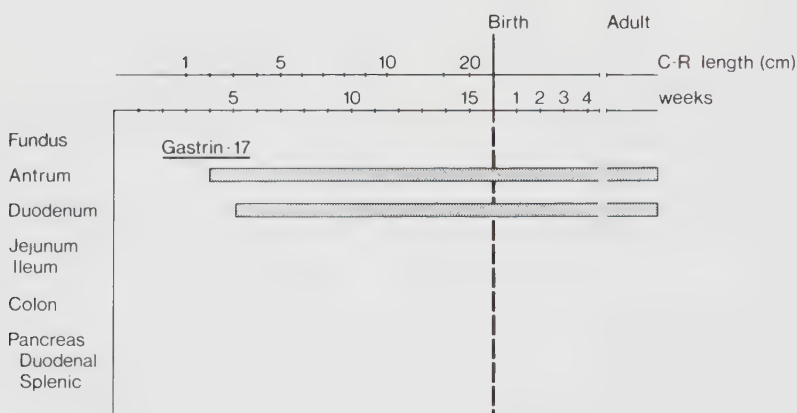
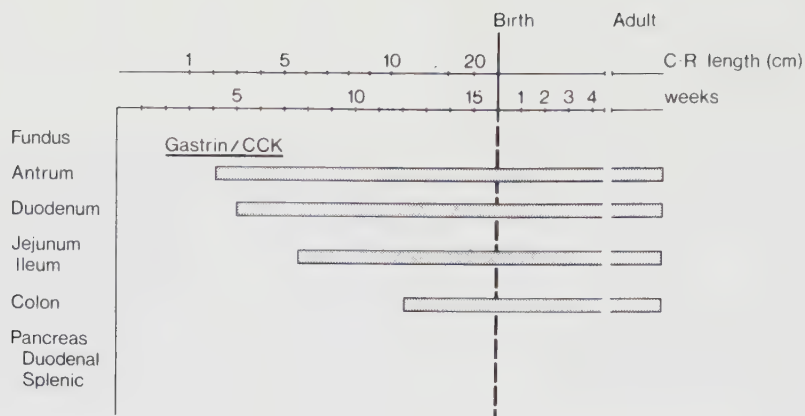


Fig. 2. Time and sites of appearance of cells storing gastrin/CCK, gastrin-17 and CCK in the gut of the fetal and newborn pig.

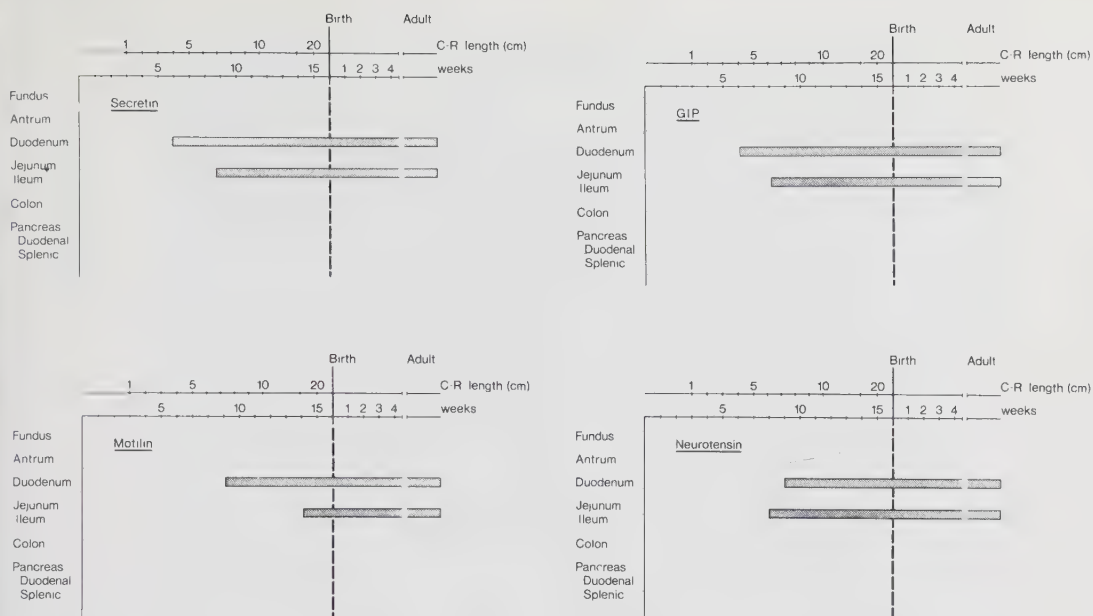


Fig. 3. Time and sites of appearance of cells storing secretin, gastric inhibitory peptide (GIP), motilin and neurotensin in the gut of the fetal and newborn pig.

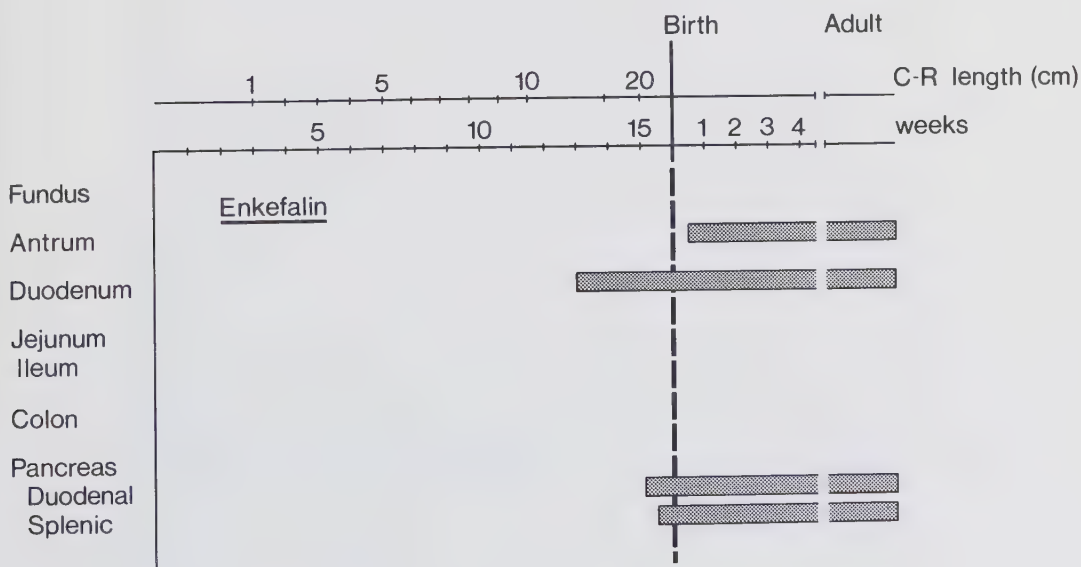


Fig. 4. Time and sites of appearance of cells storing immunoreactive enkephalin in the gut and pancreas of the fetal and newborn pig.

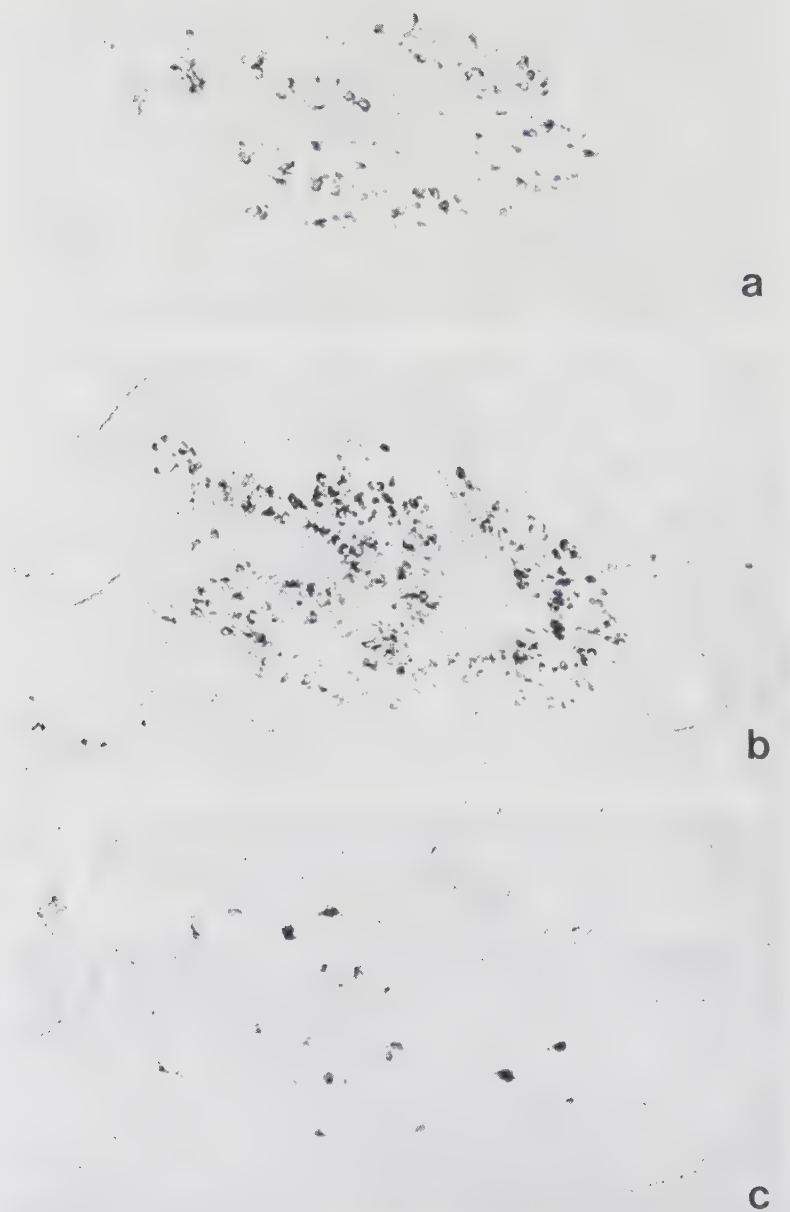


Fig. 5. Consecutive sections through the dorsal pancreatic primordium at the four week stage. The sections were immunostained for insulin (a), glucagon (b) and somatostatin (c). Glucagon cells are numerous, insulin cells moderate in number, somatostatin cells few. PAP staining (X 100).

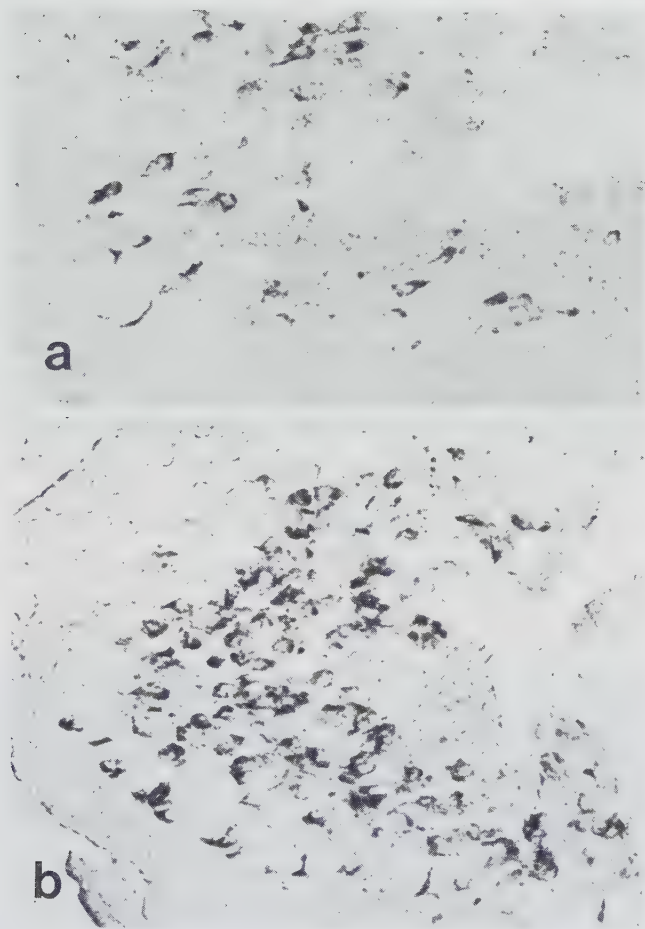


Fig. 6. Dorsal pancreatic primordium, four week stage. Sections immunostained for insulin (a) and glucagon (b). Higher magnification than in Fig. 5 to show the compact arrangement of endocrine cells. No ducts or acini are seen. PAP staining (X 250).

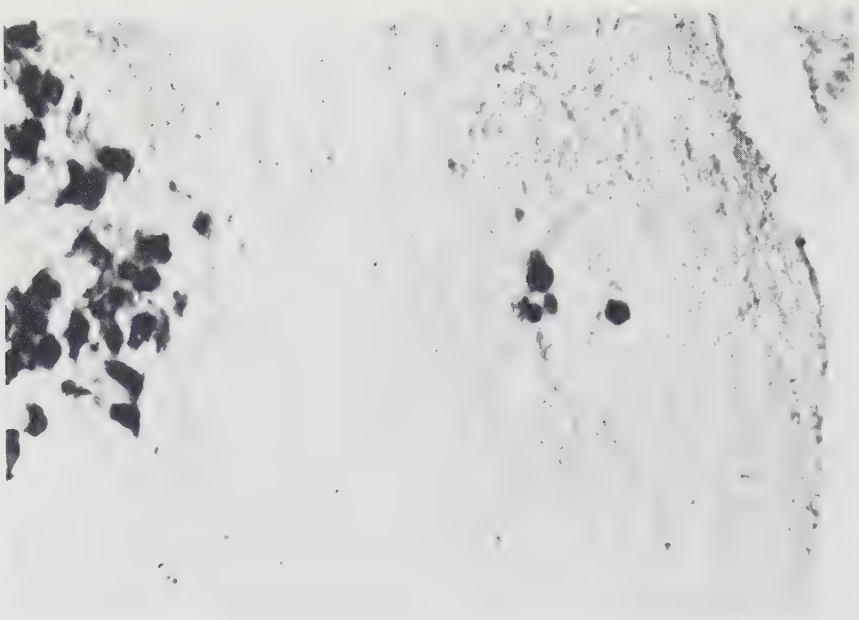


Fig. 7. Splenic portion of the pancreas, six week stage. Insulin immunofluorescence. At this stage the insulin cells are dispersed by the growing exocrine parenchyma (X 350).

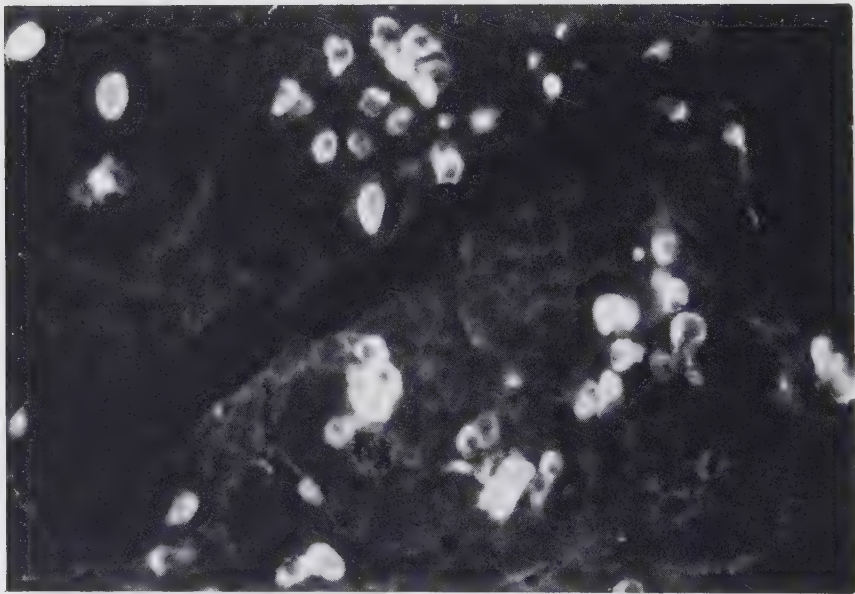


Fig. 8. Upper small intestine (right) and dorsal pancreatic primordium (left), four week stage. Glucagon cells are present in the gut epithelium and in the pancreatic primordium. PAP staining (X 300).

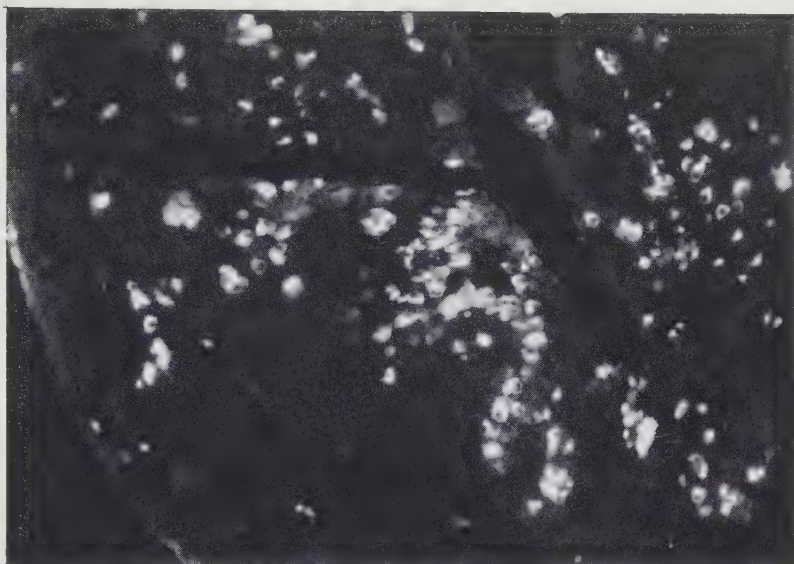


Fig. 9. Splenic portion of the pancreas, eight week stage. Glucagon immunofluorescent cells occur as single cells or nests of cells in the exocrine parenchyma (X 150).

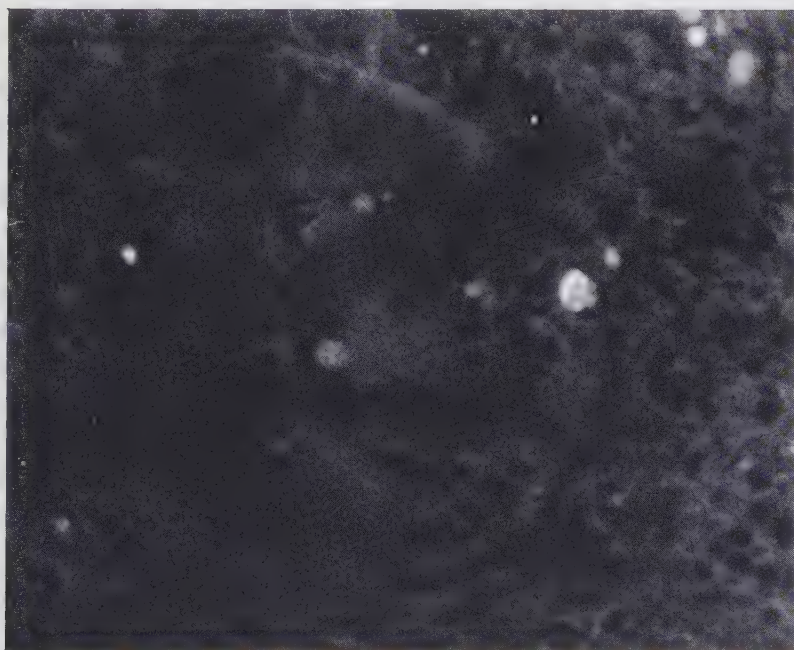


Fig. 10. Duodenum, cross-section, four week stage. Basally located glucagon immunofluorescent cell (X 400).



Fig. 11. Splenic portion of the pancreas, nine week stage. Scattered somatostatin cells. PAP staining (X 200).



Fig. 12. Gastric antrum, five week stage. Two somatostatin cells, one of which is provided with a basal process, in the epithelium. PAP staining (X 400).

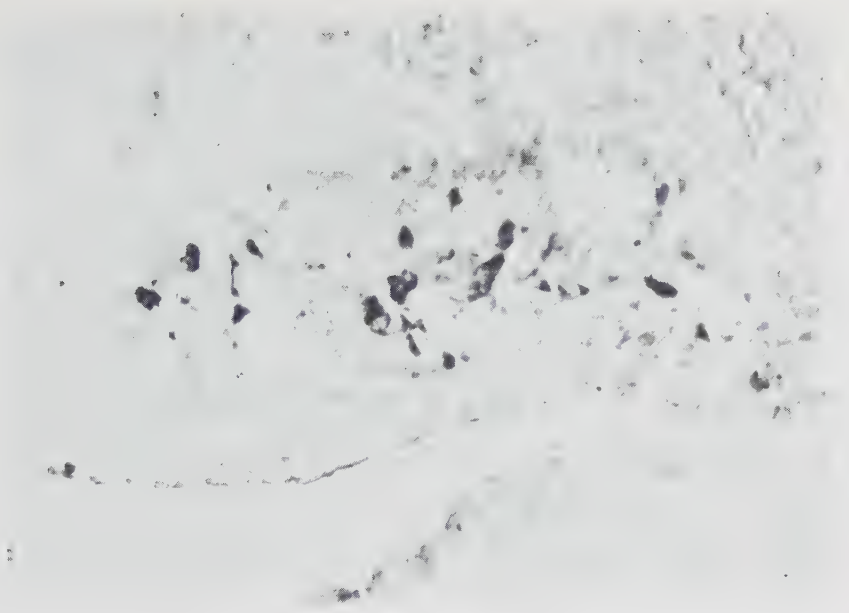


Fig. 13. Ventral pancreatic primordium, four week stage. PP cells occur scattered in the primordium. PAP staining (X 200).



Fig. 14. Antral mucosa, six week stage. Several PP immunofluorescent cells in the basal part of the epithelium (X 350).

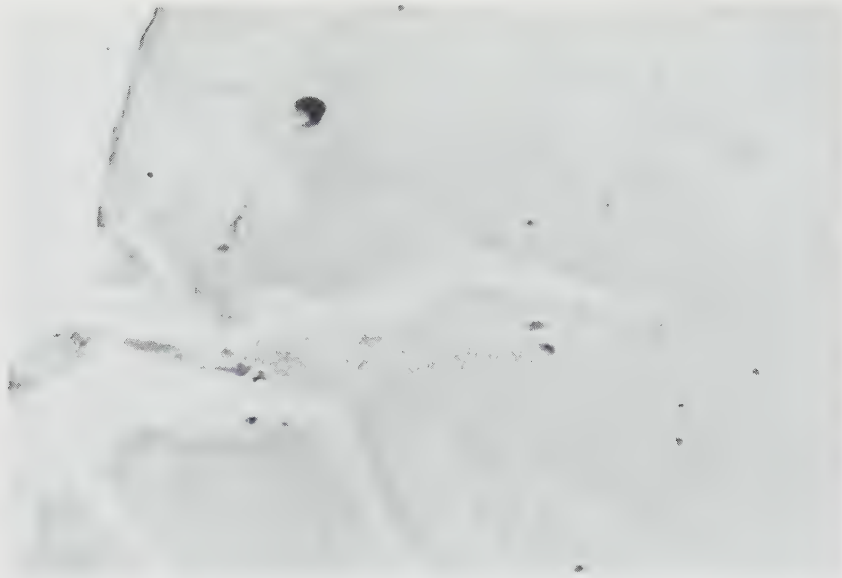


Fig. 15. Duodenum, eight week stage. Single secretin cell basally in the epithelium. PAP staining. (X 200).

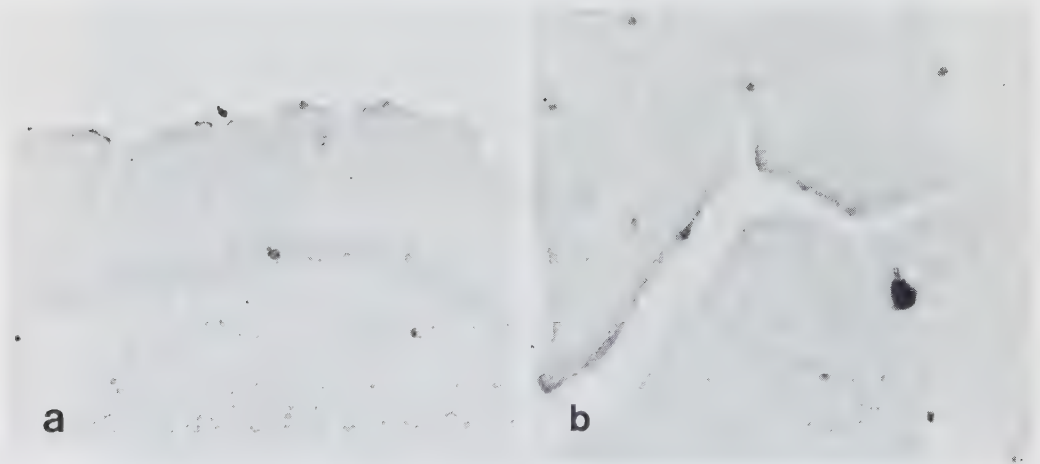


Fig. 16. (a) Gastric antrum, five week stage. Single cell displaying weak gastrin-17 immunoreactivity (X 250). (b) Duodenum, eight week stage. Single gastrin-17 storing cell in the epithelium. PAP staining (X 250).

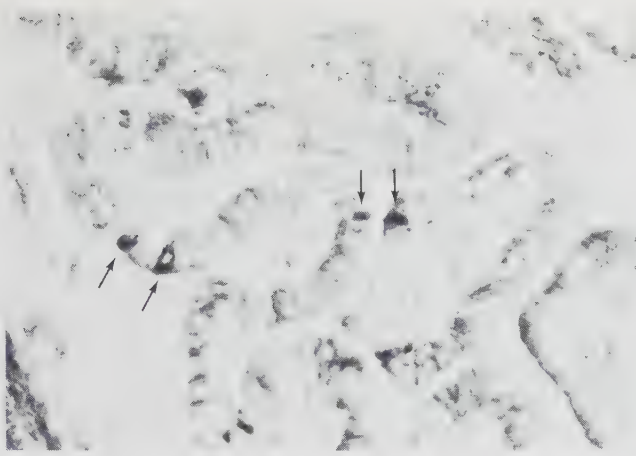


Fig. 17. Duodenum, ten week stage. Several CCK cells (arrows) in the epithelium. PAP staining (X200).

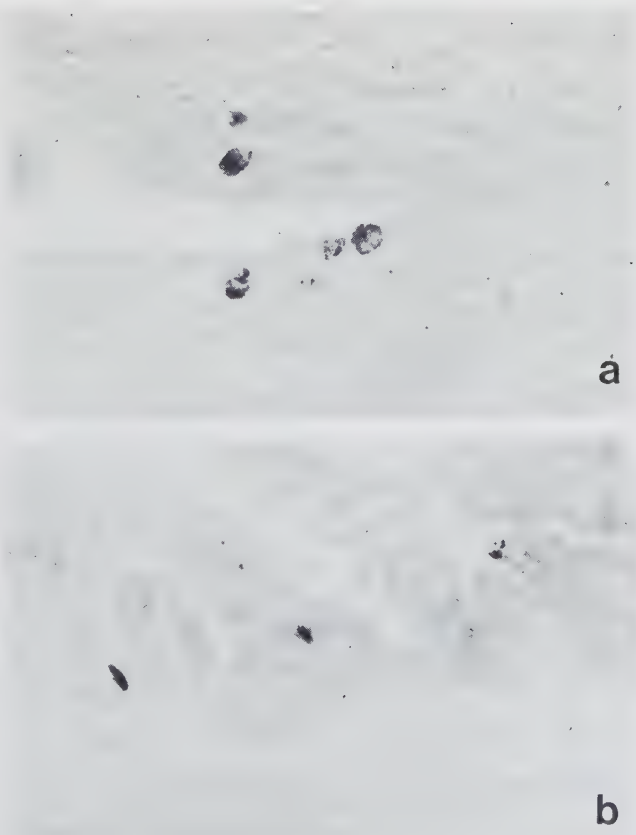


Fig. 18. Upper small intestine. (a) Six week stage. Several GIP cells in the epithelium (X 350). (b) Ten week stage. GIP cells in the crypt epithelium (X 200).

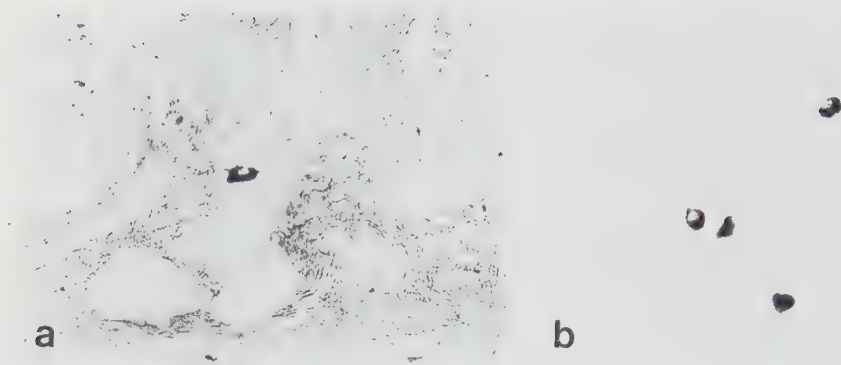


Fig. 19. (a) Gastric antrum, one week after birth. Single enkephalin cell in the epithelium (X 300).
 (b) Pancreas, duodenal portion. Enkephalin cells in the exocrine parenchyma at birth. PAP staining (X 250).

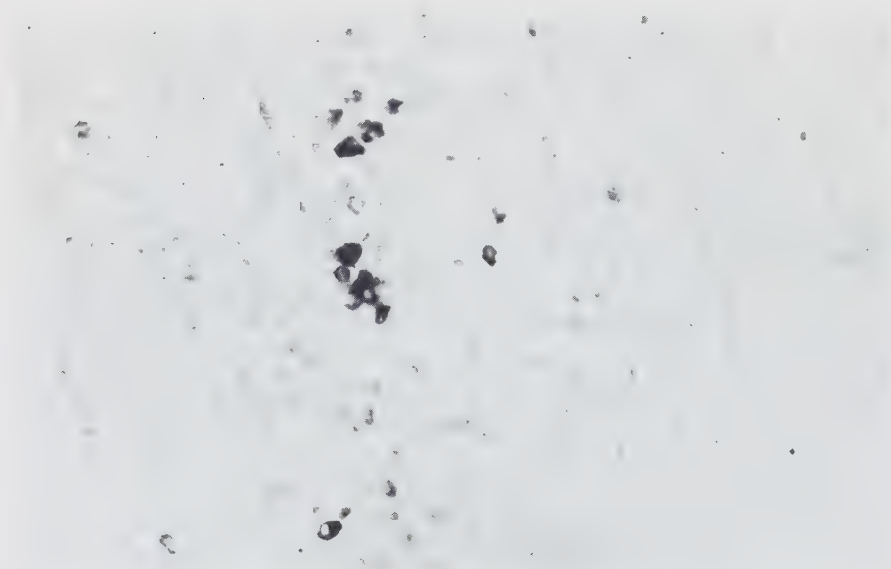


Fig. 20. Pancreas, fifteen week stage. Clusters of cells stained with antiserum against porcine ACTH (see text). The immunoreactive cells are distinct from known endocrine cell types in the pancreas. PAP staining. (X 200).

NEUROHORMONAL PEPTIDES IN ENDOCRINE TUMORS OF THE PANCREAS,
STOMACH, AND UPPER SMALL INTESTINE

An immunohistochemical study of 27 cases ^{x)}

Jan Alumets, MD, Frank Sundler, MD, PhD, Sture Falkmer,
MD, PhD, Rolf Håkanson, MD, PhD, Otto Ljungberg, MD, PhD,
Hans Mårtensson, MD, and Anders Nobin, MD, PhD

From the Departments of Histology, Pathology (at Malmö
General Hospital), Pharmacology, and Surgery, University
of Lund, Lund and Malmö, Sweden

^{x)} Presented in part at the Third International Symposium
on Gut Hormones in Cambridge, England, September 14-18,
1980. (4)

Preliminary observations have indicated the existence of characteristic spectra of gastro-entero-pancreatic (GEP) neuro-hormonal peptides in endocrine tumors arising in foregut, midgut, and hindgut derivatives. In order to further explore this feature of foregut endocrine neoplasms islet cell tumors from 14 patients were studied as well as endocrine tumors of the stomach, duodenum, and jejunum from six, five and two patients, respectively. All tumors were examined immunohistochemically with antisera raised against the islet hormones (insulin, somatostatin, glucagon, PP), the peptides of the gastrin family (gastrin, CCK), those of the secretin family (secretin, and VIP), substance P, neurotensin, leu-enkephalin, β -endorphin, motilin, calcitonin, and ACTH. In addition, one tumor was also studied ultrastructurally. The immunohistochemical observations were correlated with the clinical presentations and the results of radioimmunochemical assays of GEP neurohormones in the blood.

Virtually all the pancreatic tumors were found to be multihormonal with PP as the most common immunoreactive peptide, followed in incidence by somatostatin, insulin and glucagon. VIP, substance P, and ACTH immunoreactive cells also occurred. Of the six gastric tumors only three contained immunoreactive cell populations; gastrin cells predominated in all three. One was bihormonal (containing in addition somatostatin cells), and two monohormonal. One of the non-immunoreactive tumors was ultrastructurally found to be composed of ECL cells. Of the eight duodeno-jejunal tumors, six contained immunoreactive cells; two were bihormonal (gastrin and somatostatin) and four monohormonal (somatostatin, PP or gastrin).

In 13 of the 27 patients overt signs of endocrine disorder were observed clinically, in most cases associated with elevated peptide hormone levels in the blood. Of these 13 patients 10 had their tumors in pancreas, two in stomach and one in duodenum.

The pattern of immunoreactive neurohormonal peptides and the clinical picture were those to be expected in endocrine tumors arising in foregut derivatives. Some principles are proposed for the classification of GEP endocrine tumors on the basis of their histopathological growth pattern and spectrum of neurohormonal peptides as well as by their clinical manifestations.

INTRODUCTION

Endocrine tumors may arise in all parts of the digestive tract^{x)} (30,43). They are most commonly found in the ileum and appendix, where they originate from enterochromaffin cells; consequently, the tumor cells are argentaffin and rich in 5-hydroxytryptamine (5-HT) (16,21,42). Many ileal endocrine tumors contain not only 5-HT but also immunoreactive substance P (5,24,60). This is not surprising since in the normal gut substance P occurs in a population of 5-HT-storing enterochromaffin cells (27,56,57).

In hindgut derivatives, notably the rectum, endocrine tumors are less common. These latter tumors are usually composed of argyrophil, non-argentaffin cells (42,50,62). They have recently been shown by immunohistochemistry to harbor a variety of neurohormonal peptides, such as somatostatin, glucagon, pancreatic polypeptide (PP), substance P and enkephalin (1,20,61).

Among foregut derivatives, the pancreas and the stomach are the most common sites of endocrine tumors, whereas such tumors are comparatively rare in the duodenum and jejunum (30,43). Pancreatic endocrine tumors may cause serious metabolic and other disturbances and have therefore been studied in great detail with respect to their neurohormonal peptide content (11,15,44). Much less is known of neurohormonal peptides in tumors arising in the stomach, duodenum, and upper jejunum. This is so, not only because such tumors are rare, but also because they only occasionally give rise to symptoms of endocrine disorder (59).

The present report describes the occurrence of various neurohormonal peptides in endocrine tumors of the pancreas, stomach and upper small intestine, as revealed by immunohistochemical techniques.

^{x)} Such tumors are often referred to as carcinoid tumors, irrespective of the presence or absence of 5-hydroxytryptamine.

MATERIAL AND METHODS

Specimens of the following endocrine tumors from 27 patients were studied: fourteen from pancreas (three of them consisted of multiple nodules), six from stomach, five from duodenum, and two from proximal jejunum. Data on the patients, the localization of the tumors, and their histopathological characteristics, are given in Table 1. Data on peptide hormone levels in plasma and serum were available in a limited number of cases only (Table 1).

Most of the pancreatic tumors and some of the gastric tumors were collected at operation and specimens were immediately frozen, freeze-dried, fixed by exposure to gaseous formaldehyde⁽⁹⁾ or diethylpyrocarbonate⁽⁴⁶⁾ and embedded in paraffin *in vacuo*. These fixation procedures are superior to routine formalin fixation for the immunohistochemical demonstration of neurohormonal peptides. The duodenal endocrine tumors, obtained through the courtesy of the Cancer Registry of the National Board of Health and Welfare in Sweden, represent all such tumors available at Swedish pathology laboratories. These specimens consisted of paraffin embedded tissue blocks of routinely formalin fixed material, obtained at surgical operation or at autopsy. Poorly fixed material, as judged from sections stained with hematoxylin and eosin, was excluded. Also the jejunal endocrine tumors were routinely fixed in formalin. They were available at the Departments of Pathology in Lund and Malmö. Staining for argyrophil or argentaffin cells was performed by the conventional Grimelius⁽²³⁾ and Masson-Hamperl procedures, respectively, as described in a previous report⁽¹⁾. The presence of 5-HT was ascertained by its yellow formaldehyde-induced fluorescence⁽⁹⁾. Due to high non-specific background fluorescence in specimens stored for a long period of time the presence of 5-HT in some of the tumors could not be evaluated.

One tumor (case 17) was examined by conventional transmission electron microscopy. Small specimens were cut out from previously formalin-fixed, paraffin-embedded material and refixed

in 2.5% glutaraldehyde in 0.075 M phosphate buffer, pH 7.2. The specimens were postfixated with osmium tetroxide, dehydrated, contrasted with phosphotungstic acid and uranyl acetate, and embedded in Epon. Ultrathin sections were contrasted with lead citrate and uranyl acetate and examined in a Zeiss EM-9 electron microscope.

For the immunohistochemical investigations deparaffinized sections (6-8 μ m) were hydrated by passage through a series of ethanol and rinsed overnight in 0.1 M phosphate buffer, pH 7.2. They were then processed for the immunohistochemical demonstration of a variety of neurohormonal peptides, using either the indirect immunofluorescence method ⁽¹⁴⁾ or the immunoperoxidase (PAP) technique ⁽⁵²⁾. Details on the antisera used are given in Table 2. Controls were run as recommended ⁽⁵²⁾ and included sections incubated with antiserum inactivated by the addition of antigen (pure natural or synthetic peptide) in excess (10-100 μ g per ml diluted antiserum). Immunofluorescence was examined in a fluorescence microscope equipped with epi-illumination and with filters selected to give peak excitation at 490 nm.

RESULTS

The results of the immunohistochemical investigations are summarized in Table 3. As a rule (17 of the 27 cases), the tumor parenchyma contained more than one immunoreactive cell type. The cells were often topographically non-uniformly distributed within the tumor parenchyma; consequently, the incidence figures in Table 3 may be subject to errors, due to a patchy distribution of the immunoreactive cells.

Pancreatic tumors

All pancreatic tumors examined, except one nodule from a case of multiple endocrine neoplasia (case 7), contained immunoreactive cells (Table 3). Fifteen tumors were multihormonal in that they contained more than one immunoreactive cell population. Five tumors harbored four-different immunoreactive cell populations, five had three, five had two, and two had one.

PP was the most common immunoreactive peptide, present in 11 of the 18 tumors. Next in incidence was somatostatin, present in nine tumors. Immunoreactive substance P, neurotensin, leu-enkephalin, calcitonin, and ACTH were found in one tumor each.

Ten of the 18 tumors contained one predominating immunoreactive cell population. This majority cell population consisted of insulin cells in three tumors (cases 1, 2 and 6), somatostatin cells in two (cases 5 and 10), glucagon cells in one (case 9), PP cells in two (cases 3 and 4), gastrin cells in two (cases 8 and 13), and VIP cells in one (case 12). In one of the three insulin-producing tumors (case 2) the insulin cells contained, in addition, immunoreactive calcitonin ⁽⁵⁴⁾ (Fig.1).

All tumors containing insulin, glucagon, gastrin, or VIP immunoreactive cells as majority cell populations, gave rise to the expected clinical manifestations ^(33,45,48,64) (Table 1).

Neither of the two tumors having PP cells as the majority cell population was associated with symptoms of endocrine disorder. Of the two tumors with somatostatin cells as majority cell population (Fig. 2), one (case 10) was associated with mild diabetes mellitus, whereas the other (case 5) was associated with peptic ulcer, conceivably because the tumor harbored a minority gastrin cell population, causing hypergastrinemia (Table 1).

Three of the four patients suffering from multiple endocrine neoplasia(MEN), type I ⁽⁴⁹⁾ (Table 1) had multiple endocrine tumors in the pancreas (cases 6, 7 and 12). Three of the larger tumor nodules were examined from each of case 6 and 7, whereas only the largest one from case 12 was studied. The spectrum of immunoreactive peptides differed from one nodule to another (Fig. 3). Glucagon cells were the majority cell population in one nodule (case 6), insulin cells in another (case 6), and VIP cells (Fig. 4) in still another (case 12).

In addition, several neurohormonal peptides occurred in minority cell populations (Table 3). One of the tumor nodules (case 7) did not contain any demonstrable peptide. The presence of argyrophil cells in the tumors (Table 1) generally re-

flected the spectrum of immunoreactive peptides observed (Table 3). Thus, most cells in those tumors storing insulin, somatostatin or PP, were non-argyrophil or weakly argyrophil, whereas those containing glucagon, gastrin or VIP stained strongly. Despite the presence of 5-HT-fluorescent cells in several of the tumors, no argentaffin cells were observed (Table 1).

Gastric tumors

Three of the six gastric tumors (cases 15, 16 and 19) contained immunoreactive cell populations; gastrin cells predominated in all three. One pyloric tumor (case 19), containing gastrin cells as the majority cell population, gave rise to hypergastrinemia symptoms and was associated with pancreatic nesidoblastosis⁽³⁷⁾. The other two tumors (cases 15 and 16), having gastrin cells as the predominating cell type (Fig. 5), were not associated with symptoms of hypergastrinemia. One (case 15) was a small tumor in the pyloric region, the other (case 16) was conspicuously large and located in the corpus region of the stomach.

The three tumors devoid of immunoreactive cells were classified as endocrine neoplasms by their light-microscopic appearance and the presence of argyrophil tumor cells (cases 16, 17 and 19), the characteristic ultrastructure (case 16), and the occurrence of symptoms of endocrine disorder (case 17). No argentaffin cells were found in any of these gastric tumors. One tumor (case 17) in the corpus region was associated with severe atrophic gastritis and widespread intestinal metaplasia. By electron microscopy, it was found to be composed of endocrine cells of the ECL type^(25,51). Another (case 18) was a large neoplasm in the corpus region, associated with Cushing's syndrome. It was also exceptional in displaying not only argyrophil but also argentaffin, 5-HT-containing, tumor cells (Table 1). However, ACTH immunoreactive cells could not be demonstrated, neither in the primary tumor, nor in its metastases. Finally, one tumor in the corpus (case 20) was not only devoid of immunoreactive cells, but also of any association with overt clinical symptoms. It contained, however, numerous

argyrophil tumor cells and was light-microscopically assessed as an endocrine neoplasm.

Duodeno-jejunal tumors

Six of the seven tumors contained immunoreactive cell populations. The remaining one was classified as endocrine neoplasm because of its characteristic light-microscopical appearance with occurrence of argyrophil cells. No argentaffin cells were found in any of the duodeno-jejunal tumors (Table 1).

Two of the tumors (cases 22 and 26) containing immunoreactive cells were bihormonal and associated with peptic ulcer. One (case 22) was a duodenal tumor composed predominantly of gastrin cells (Fig. 7) with somatostatin cells as a minority cell population. The other (case 26) was a jejunal tumor composed predominantly of somatostatin cells and only few gastrin cells. In each of the remaining four tumors only one cell population could be demonstrated, storing somatostatin (case 21) (Fig.7), PP (case 23), or gastrin (cases 24 and 27). In each of these tumors the immunoreactive cells constituted the majority cell population.

DISCUSSION

The present findings support the view that gastro-entero-pancreatic (GEP) endocrine tumors often are multihormonal (15,16, 34). This was particularly evident in the pancreatic tumors, but one of the six gastric tumors and two of the seven duodeno-jejunal ones also contained more than one immunohistochemically demonstrable endocrine cell type.

The immunohistochemical observations indicated that endocrine tumors in the pancreas, stomach, and duodeno-jejunum, have traits in common. Thus, they contained a spectrum of neurohormonal peptides, resembling that found normally in these regions, including insulin, somatostatin, glucagon, PP, and gastrin. Nonetheless, tumors arising in different foregut derivatives seem to differ from each other, for instance in that insulin and glucagon cells are more or less restricted to tumors

of the pancreas ⁽¹⁵⁾, whereas ECL cells are found exclusively in tumors of the stomach ^(11,38,51,59). In tumors arising in the upper small intestine, somatostatin and gastrin immunoreactive cells may occur together in varying proportions, from those apparently containing only somatostatin immunoreactive cells at one extreme to those seemingly composed of gastrin cells exclusively at the other ^(2,17,31,60). With regard to the spectrum of neurohormonal peptides, foregut tumors collectively resemble hindgut endocrine tumors while being different from those of the midgut ^(1,4,20,62). Immunoreactive peptides common to foregut and hindgut tumors are somatostatin, glucagon, and PP. Insulin, gastrin, and VIP, however, are rarely found in hindgut tumors ⁽¹⁾. Preliminary observations suggest that the immunoreactive glucagon ⁽¹³⁾ in pancreatic tumors represents chiefly pancreatic-type glucagon, whereas in hindgut tumors it is predominantly gut-type glucagon (glicentin) (own unpublished observations).

In view of the well-known heterogeneity of gastrin ⁽⁴⁷⁾ and its close chemical relationship with CCK, an attempt was made to differentiate between CCK and members of the gastrin family by immunohistochemical analysis. All available gastrin tumors displayed gastrin-17 and gastrin-34 immunoreactivity, whereas none displayed CCK immunoreactivity. This was characteristic also of the gastrin tumors not evoking hypergastrinemia symptoms.

Foregut endocrine tumors, particularly those in the pancreas, are often associated with clinical symptoms of hormone/^{over-}production ⁽²⁸⁾. Typical examples are those secreting insulin ⁽³³⁾, glucagon ^(10,40,48), gastrin ⁽⁶⁴⁾, and VIP ⁽⁴⁵⁾. By contrast, hindgut endocrine tumors, although rich in immunoreactive neurohormonal peptides, seem never to cause symptoms of hormone overproduction ⁽¹⁾.

Somatostatin producing tumors are thought to give rise to a specific constellation of symptoms, "somatostatinoma syndrome", such as mild diabetes mellitus and steatorrhea ⁽³⁶⁾. Neither of the two tumors composed chiefly of somatostatin cells

(cases 5 and 21) were associated with symptoms of the "somatostatinoma syndrome". Also the PP cell tumors (cases 3, 4 and 23) did not seem to cause specific clinical symptoms. Conceivably, somatostatin and PP producing tumors constitute a significant fraction of "non-functional" pancreatic endocrine tumors (32,48).

To judge from the present observations and from published reports, GEP endocrine cells vary in their tendency to give rise to tumors. Thus, certain cell types, e.g. those producing CCK, secretin, or motilin, do not seem to cause tumors, whereas other cell types, e.g. those producing insulin or gastrin, do so comparatively often. Some of the GEP endocrine tumors, e.g. those producing insulin, glucagon/glicentin, or PP, commonly arise in locations where their anticipated progenitor cells occur normally (17,18). Others, e.g. those producing gastrin seem to arise preferentially in locations where the progenitor cells is not found normally. The progenitor cell of VIP-producing endocrine tumors is unknown. Interestingly, the VIP antiserum used in the present study readily demonstrates nerve fibers but does not detect endocrine cells in the mammalian gut and pancreas. This observation has led to the conclusion that VIP is a neuropeptide rather than a hormone (34). Consequently, the cell of origin of VIP-producing tumors is not readily identified. In birds (55) and cartilaginous fish (19) VIP occurs in endocrine cells in the gut. Conceivably, the occurrence of VIP cells in GEP tumors may reflect the phylogeny of such cells (18).

With the advent of immunohistochemical techniques and with increased knowledge of the ontogeny (7) and phylogeny (18) of the GEP neuroendocrine system, new means have become available to differentiate histopathologically between endocrine tumors in various parts of the gastrointestinal tract and pancreas and to understand their histogenesis and biological properties.

The results of the present study highlights the problem of classifying endocrine tumors, especially those containing more

than one endocrine cell type. In view of the fact that such tumors (e.g. hindgut tumors) are often not associated with specific symptoms ^(1,22), it seems preferable to assess them primarily by their cellular composition. The presence of argyrophil tumor cells is obviously of great aid for the detection of endocrine GEP tumors. Thus, we suggest that a reasonable solution of the classification problem is to characterize a GEP endocrine tumor on the basis of the following histopathological and clinical features:

1. Its histopathology (degree of differentiation, invasive growth, metastases), including information on the presence or absence of argentaffin and/or argyrophil cells;
2. Its site of origin;
3. Its spectrum of GEP neurohormonal peptides;
4. Its association with signs or symptoms of endocrine disorder.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Cancer Society (Project No. 805-05xA), the Swedish Research Council (Projects Nos. 04x4499 and 12x718), and the Cancer Research Foundation at Malmö General Hospital.

TABLE 1. Age, sex, tumor size, histological type, occurrence of argyrophil/argentaffin and 5-HT-containing tumor cells and some clinical aspects of the 27 patients with endocrine tumors of foregut origin.

Tumor site	Case no	Age/Sex	Tumor nodule no	Tumor size (cm)	Histol. type ^a	Argyro-phil cells ^b	Argen-taffin cells ^c	5-HT containing cells	Clinical presentation
Pan-creas	1	69 F		1	E(A+B)	+	-	-	Hyperinsulinism with hypoglycemia.
	2	41 F		1	E(A+C9)	+	-	-	Hyperinsulinism with hypoglycemia; hypercalcitoninemia (56).
	3	56 F		n.i.	E(A+C)	+	-	+	No signs or symptoms of endocrine disorder; elevated plasma levels of PP and 5-HT.
	4	67 F		1	E	+	-	+	No signs or symptoms of endocrine disorder.
	5	52 M		n.i.	D	+	-	-	Hypergastrinemia and peptic ulcers but no full-blown Zollinger-Ellison syndrome (65). Liver and lymph node metastases.
	6	30 F	I II III	1-2	E(B+C)	+	-	-	Multiple endocrine neoplasia, type I (49). Daughter to patient no. 12. Multiple tumor nodules in pancreas; three of them studied.
	7	41 M	I II III	1-2	E(A+C)	+	-	(+) (+) (+)	Multiple endocrine neoplasia, type I (49), including also a Zollinger-Ellison syndrome (65) with hypergastrinemia and jejunal peptic ulcers. Hyperparathyroidism with hypercalcemia. Multiple tumor nodules in pancreas; three of them studied.
	8	55 M		n.i.	E(A+C)	+	-	n.t.	Multiple endocrine neoplasia, type I (49), including Zollinger-Ellison syndrome (65) with hypergastrinemia. Father to patient no. 13.
	9	72 M		3	C	+	-	n.t.	Glucagonoma syndrome (28) with hyperglycemia.
	10	86 M		1	C	+	-	n.t.	Mild diabetes mellitus. Tumor found incidentally at autopsy.

TABLE 1 (continued)

Tumor site	Case no	Age/Sex	Tumor nodule no	Tumor size (cm)	Histol. type ^a	Argyro-phil cells ^b	Argen-taffin cells ^c	5-HT containing cells	Clinical presentation
	11	56 M		2	A	+	-	n.t.	No signs or symptoms of endocrine disorder. Lymph node metastases.
	12	53 M		10	D	+	-	n.t.	Multiple endocrine neoplasia, type I (49) including Verner-Morrison syndrome (45) with hypokalemia and diarrhea. Recurrent peptic ulcers of the stomach and duodenum. Hyperparathyroidism with hypercalcemia. Multiple tumor nodules in pancreas; only one of them studied. Liver metastases. Father to patient no. 6.
	13	36 F		n.i.	E	+	-	n.t.	Zollinger-Ellison syndrome (65) with hypergastrinemia. Daughter to patient no. 8.
	14	68 M		5	B	+	-	n.t.	Glucagonoma syndrome; hyperglucagonemia.
Stomach	15	79 M		1	C	+	-	n.t.	No signs or symptoms of endocrine disorder. Pyloric tumor, found incidentally at autopsy.
	16	65 F		5	E(B+C)	+	-	-	No signs or symptoms of endocrine disorder. Large tumor in the corpus region of the stomach.
	17	26 F		1	C	+	-	n.t.	Achylia and atrophic gastritis. Gastrin cell proliferation with hypergastrinemia. Intestinal metaplasia of the stomach mucosa around the tumor in the corpus region. Thyroid hypofunction.
	18	36 F		n.i.	D	+	+	(+)	Elevated plasma levels of cortisol; features of Cushing's syndrome. Large tumor in the corpus region of the stomach. Liver and lymph node metastases.

19	65	M	1	E(A+B)	+	-	-	Hypergastrinemia with peptic ulcer. Tumor in the antrum-pyloric region (37).
20	75	M	n.i.	D	+	-	n.t.	No signs or symptoms of endocrine disorder. Tumor in corpus region of the stomach with lymph node metastases.
21	68	M	n.i.	D	+	-	n.t.	No signs or symptoms of endocrine disorder. Tumor in proximal duodenum.
22	59	F	n.i.	E	+	-	n.t.	Hypergastrinemia but no ulcer. Tumor in proximal duodenum.
23	50	M	1	D	-	-	-	No signs or symptoms of endocrine disorder, except for rising concentrations of PP in the blood one year after operation. Tumor near papilla Vateri (41).
24	36	F	2	B	+	-	n.t.	No signs or symptoms of endocrine disorder. Tumor in proximal duodenum.
25	69	F	n.i.	E(A+C)	+	-	n.t.	No signs or symptoms of endocrine disorder. Tumor in distal duodenum.
26	55	M	1	E(A+C)	+	-	-	Recurrent peptic ulcers. Tumor in proximal jejunum. Liver and lymph node metastases (21).
27	58	F	1	A	+	-	n.t.	No signs or symptoms of endocrine disorder. Tumor in proximal jejunum. Liver and lymph node metastases.

a) according to Soga (50): A = "solid", B = "trabecular", C = "tubular", D = "undifferent", E = "mixed types".

b) Grimelius' technique (23).

c) Masson Hamperl technique (23).

+ and - denote presence and absence, respectively, of argyrophil or argentaffin material or 5-HT in tumor cells.

n.t. = not tested due to unsatisfactorily fixed or stored material. n.i. = no information available.

TABLE 2. Source and working dilutions of antisera giving immunostaining of the present series of endocrine foregut tumors. Antisera, raised against secretin, CCK, β -endorphin, and motilin, gave no immunostaining.

Antisera against	Working dilution				Refs.
	Antigen	Immunofluorescence	PAP staining	Code	
Insulin	Pure bovine insulin	1/80	1/5120	LA 1	L.G. Heding, Novo Res.Inst. Bagsvaerd, Denmark (26)
Somatostatin	Synthetic ovine somatostatin	1/40	1/1280	19578	M.P. Dubois, Station Physiol. Reprod., INRA, Jouzilly, France (8)
Glucagon	1) Pure porcine pancreatic glucagon	1/160	1/640	7811 ^{a)}	Milab, Malmö, Sweden
	2) Pure porcine pancreatic glucagon	1/160	1/640	7810 ^{b)}	Milab, Malmö, Sweden
Pancreatic Polypeptide (PP)	Pure human PP	1/80	1/2560	HPP	R.E.Chance, El.Lilly, Res.Lab., Indianapolis, Ind., USA (39)
Gastrin	1) Synthetic human gastrin 2-17	1/320	1/5120	4562 ^{c)}	J.F. Rehfeld, Dept.Med.Chem., Univ. Aarhus, Denmark (35)
	2) Synthetic human gastrin 1-17	1/10	1/320	L 6 ^{d)}	G.J.Dockray, Dept.Physiol., Univ., Liverpool, England (58)
	3) Synthetic N-terminal fragment of "big gastrin"	1/40	1/640	R2702 ^{e)}	N. Yanaihara, Dept.Bioorganic Chem., College Pharmacy, Shizuoka, Japan (29)
Vasoactive Intestinal Polypeptide (VIP)	Pure porcine VIP		1/5120	7852	Milab, Malmö, Sweden
Substance P	Synthetic bovine substance P	1/20	1/160	SP 8 ^{f)}	P.C.Emson, Med.Res.Council, Neurochem. Unit, Univ., Cambridge, England (12)

Neurotensin	Synthetic neurotensin	1/40	1/640	HC-8	R.E. Carraway, Dept. Phys. and Lab. Human Reprod., Harvard Med. Sch., Boston, Mass., USA	(7)
Enkephalin	Synthetic leu-enkephalin	1/60	1/240	"leu-enk"	R.J. Miller and K.-J. Chang, Wellcome Res. Lab., Burroughs Wellcome Co., Triangle Park, N.C., USA	(1)
Calcitonin	Synthetic human calcitonin	1/80	1/640	AS 292/5	I. MacIntyre, Endocrine Unit, Royal Postgrad., Med. Sch., London, England	(6)
Adrenocorticotrophic hormone (ACTH)	Pure porcine ACTH 1-39	1/80	1/640	19)	Own	(53)

IX:17

-
- This antiserum cross-reacts with glicentin (gut-type glucagon).
 - This antiserum is specific for pancreatic-type glucagon.
 - This antiserum is directed against the COOH-terminal for amino acids, common to gastrin and cholecystokinin (CCK) and cross-reacts with CCK.
 - This antiserum is specific for gastrin-17 and does not cross-react with CCK.
 - This antiserum is specific for "big gastrin".
 - This antiserum cross-reacts with physalaemin.
 - This antiserum contains also antibodies directed against a contaminant in the ACTH preparation used for immunization.

TABLE 3. Immunohistochemical demonstration of neurohormonal peptides in the present series of foregut endocrine tumors. Antisera, raised against secretin, CCK, β -endorphin, and motilin, gave no immunostaining.

Tumor site	Case no.	Tumor nodule no.	Occurrence of tumor cells, immunoreactive with antisera against												
			Insulin		Somato-	Glucagon		Gastrin			Substance P	Neuro-	Leu-en-	Calci-	
			1	2	statin	1)	2)	1)	2)	3)	P	tensin	kephalin	tonin	ACTH
Pancreas	1		+++	-	+	-	-	-	-	-	-	-	-	-	-
	2		+++	-	-	-	+	-	-	-	-	-	++	-	-
	3		-	-	-	-	-	+++	-	-	-	-	-	-	-
	4		-	-	+	-	-	+++	-	-	-	-	-	-	-
	5		-	-	+++	-	-	++	+	+	-	-	-	-	-
	6	I	+++	-	+	-	-	+	-	-	-	-	-	-	-
		II	-	-	-	++	-	++	-	-	+	-	-	-	-
		III	+	+	+	+	+	+	-	-	-	-	-	-	-
	7	I	-	-	-	-	-	-	-	-	-	-	-	-	-
		II	-	-	-	+	-	+	-	-	-	-	-	-	-
		III	-	-	+	-	-	-	+	+	-	-	-	-	-
	8		-	-	-	-	-	-	+++	++	++	-	-	-	-
	9		+	-	+	+++	-	-	-	-	-	+	-	-	-
	10		+	-	+++	++	-	-	-	-	-	-	-	-	-
	11		-	-	+	+	-	-	-	-	+	-	-	+	-
	12	I	-	-	-	-	-	++	+	-	-	+++	+	-	-
	13		-	-	-	-	-	+	+++	+	+	-	-	-	-
	14		-	++	-	++	++	-	-	-	-	-	-	-	-

Stomach	15	-	+	-	-	-	+++	+++	+++	n.t.	-	-	-	-
	16	-	-	-	-	-	+++	+++	+++	n.t.	-	-	-	-
	17	-	-	-	-	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-	-	-	-	-	-
	19	-	-	-	-	-	+++	+++	+++	n.t.	-	-	-	-
	20	-	-	-	-	-	-	-	-	-	-	-	-	-
Duodenum	21	-	+++	-	-	-	-	-	-	-	-	-	-	-
and	22	-	+	-	-	-	++	++	++	-	-	-	-	-
upper	23	-	-	-	-	+++	-	-	-	-	-	-	-	-
jejunum	24	-	-	-	-	-	+++	+++	+++	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	++	-	-	-	+	+	+	-	-	-	-	-
	27	-	-	-	-	-	+	+	+	-	-	-	-	-

The differences between the two types of glucagon antisera used and between the three gastrin antisera are given in footnotes to Table 2.

+

+

+++

-

n.t.

: denotes few immunoreactive tumor cells.

: denotes moderate numbers of immunoreactive tumor cells.

: denotes numerous immunoreactive tumor cells.

: denotes no immunoreactive cells.

: not tested

The pancreas of cases 6, 7 and 12 harbored multiple tumor nodules, three of which (I, II and III) were studied in cases 6 and 7.

REFERENCES

1. Alumets, J., Alm, P., Falkmer, S., Håkanson, R., Ljungberg, O., Mårtensson, H., Sundler, F., and Tibblin, S.: Immunohistochemical evidence of peptide hormones in endocrine tumors of the rectum. *Cancer*, (in press), 1981.
2. Alumets, J., Ekelund, G., Håkanson, R., Ljungberg, O., Ljungqvist, U., Sundler, F., and Tibblin, S.: Jejunal endocrine tumour composed of somatostatin and gastrin cells and associated with duodenal ulcer disease. *Virchows Arch.A.Path.Anat. and Histol.* 378:17-22, 1978.
3. Alumets, J., Fahrenkrug, J., Håkanson, R., Schaffalitzky de Muckadell, O., Sundler, F., and Uddman, R.: A rich VIP nerve supply is characteristic of sphincters. *Nature* 280:155-156, 1979.
4. Alumets, J., Falkmer, S., Håkanson, R., Ljungberg, O., and Sundler, F.: Characteristic spectrum of neurohormonal peptides in endocrine tumours arising in foregut, midgut or hindgut derivatives. *Regul.Pept.* 1, Suppl. 1, S3, 1980.
5. Alumets, J., Håkanson, R., Ingemansson, S., and Sundler, F.: Substance P and 5-HT in granules isolated from an intestinal argentaffin carcinoid. *Histochemistry*, 52:217-222, 1977.
6. Alumets, J., Håkanson, R., Lundqvist, G., Sundler, F., and Thorell, J.: Ontogeny and ultrastructure of somatostatin and calcitonin cells in the thyroid gland of the rat. *Cell Tissue Res.* 206:193-201, 1980.
7. Alumets, J., Håkanson, R., and Sundler, F.: Ontogeny of endocrine cells in porcine gut and pancreas. An immunocytochemical study. *Gastroenterology*, (in press), 1981.
8. Alumets, J., Sundler, F., and Håkanson, R.: Distribution, ontogeny, and ultrastructure of somatostatin immunoreactive cells in the pancreas and gut. *Cell Tiss. Res.* 185:465-479, 1977.

9. Björklund, A., Falck, B., and Owman, C.: Fluorescence microscopic and microspectro-fluorometric techniques for the cellular localization and characterization of biogenic amines. In *Methods of investigative and diagnostic endocrinology*, edit. S.A. Berson, vol. 1: *The thyroid and biogenic amines*, edit. by J.E. Rall and I.J. Kopin, PP.318-368. Amsterdam: North-Holland Publ. Comp. 1972.
10. Bordi, C., Ravazzola, M., Baetens, D., Gorden, P., Unger, G.R., and Orci, L.: A study of glucagonomas by light and electron microscopy and immunofluorescence. *Diabetes* 28:925-936, 1979.
11. Bordi, C., Senatore, S., and Missale, G.: Gastric carcinoid following gastrojejunostomy. *Am.J.Digest.Dis.* 21:667-671, 1976.
12. Brodin, E., Alumets, J., Håkanson, R., Leander, S., and Sundler, F.: Immunoreactive substance P in the chicken gut: Distribution, development and possible functional significance. *Cell Tissue Res.*, (in press), 1981.
13. Conlon, J.M.: The glucagon-like polypeptides - Order out of Chaos? *Diabetologia* 18:85-88, 1980.
14. Coons, A.H., Leduc, E.H., and Conolly, J.M.: Studies on antibody production. 1. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J.Exp.Med.* 102:49-60, 1955.
15. Creutzfeldt, W.: Endocrine tumors of the pancreas: Clinical, chemical and morphological findings. In *The Pancreas*. P.J. Fitzgerald and A.B. Morrison (eds.) Williams & Wilkins, Baltimore/London, p. 208-230, 1980.
16. Dawson, I.M.P.: The endocrine cells of the gastro-intestinal tract and the neoplasms which arise from them. *Current Topics Path.* 63:222-258, 1976.
17. DeLellis, R.A., Gagel, R.F., Kaplan, M.M., and Curtis, L.E.: Gastrinoma of duodenal G-cell origin. *Cancer* 38:201-208, 1976.

18. Falkmer, S., Carraway, R.E., El-Salhy, M., Emdin, S.O., Grimelius, L., Rehfeld, J.F., Reinecke, M. and Schwartz, T.F.W.: Phylogeny of the gastroenteropancreatic neuroendocrine system. A review with special reference to the occurrence of CCK-like and neurotensin-like polypeptides in lower vertebrates and invertebrates.
UCLA Forum Med.Sci. 23:21-42, 1981.
19. Falkmer, S., Fahrenkrug, J., Alumets, J., Håkanson, R., and Sundler, F.: Vasoactive intestinal polypeptide (VIP) in epithelial cells of the gut mucosa of an elasmobranchian cartilaginous fish, the ray.
Endocrinol.Japon.Suppl. 1:401-405, 1980.
20. Fiocca, R., Capella, C., Buffa, R., Fontana, R., Solcia, E., Hage, E., Chance, R.E., and Moody, A.J.: Glucagon-like, glicentin-like, and pancreatic polypeptide-like immunoreactivities in rectal carcinoids and related colorectal cells.
Am.J.Path. 100:81-92, 1980.
21. Grahame-Smith, D.G.: The carcinoid syndrome. In *William Heinemann Medical Books Ltd*, London, pp. 1-96, 1972.
22. Grimelius, L., Hultquist, G.T., and Stenkvist, B.: Cytological differentiation of asymptomatic pancreatic islet cell tumours in autopsy material.
Virchows Arch.A Path.Anat. and Histol. 365:275-288, 1975.
23. Grimelius, L., and Wilander, E.: Silver stains in the study of endocrine cells of the gut and pancreas.
Invest.Cell.Pathol. 3:3-12, 1980.
24. Håkanson, R., Bengmark, S., Brodin, E., Ingemansson, S., Larsson, L.-I., Nilsson, G., and Sundler, F.: Substance P like immunoreactivity in intestinal carcinoid tumours. In *Substance P*. von Euler, and B. Pernow, eds. Raven Press, New York, pp. 55-58, 1977.
25. Håkanson, R., Owman, C., Sporrang, B., and Sundler, F.: Electron microscopic identification of histamine-storing argyrophil (enterochromaffin-like) cells in the rat stomach.
Z. Zellforsch. 122:460-466, 1971.

26. Heding, L.G.: Determination of total serum insulin (IRI) in insulin-treated diabetic patients.
Diabetologia 8:260-266, 1972.
27. Heitz, P., Polak, J.M., Timson, C.M., and Pearse, A.G.E.: Enterochromaffin cells as the endocrine source of gastrointestinal substance P.
Histochemistry, 49:343-347, 1976.
28. Holst, J.J.: Gut endocrine tumour syndromes.
Clinics Endocr. Metab. 8:413-432, 1979.
29. Iwanaga, T., Kusumoto, Y., Fujita, T., Yanaihara, C., Mochizuki, T., and Yanaihara, N.: Immunocytochemical localization of the different gastrin forms in the pyloric antrum.
Biomed. Res. 1:316-320, 1980.
30. Jouanneau, P., Malafosse, M.: Les tumeurs carcinoides du tube digestif.
Monogr.Assoc.Franç.Chir. 73:1-193, 1971.
31. Kaneko, H., Yanaihara, N., Ito, S., Kusumoto, Y., Fujita, T., Ishikawa, S., Sumida, T., and Sekiya, M.: Somatostatinoma of the duodenum.
Cancer 44:2273-2279, 1979.
32. Kent, R.B., van Heerden, J.A., and Weiland, L.H.: Non-functioning islet cell tumors.
Ann.Surg. 193:185-190, 1981.
33. Klöppel, G., and Heitz, P.: Endocrines Pankreas und Diabetes mellitus. Die disseminierten (diffusen) endokrinen Zellen. In: *Spezielle pathologische Anatomie, Vol. 14, Pathologie der endokrinen Organe.* G. Seifert, Ed., Springer Verlag, Berlin-Heidelberg, pp. 523-728, and 1079-1135, 1981.
34. Larsson, L.I., Fahrenkrug, J., Schaffalitzky de Muckadell, O., Sundler, F., Håkanson, R., and Rehfeld, J.F.: Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons.
Proc.Natl.Acad.Sci. USA 73:3197-3200, 1976.
35. Larsson, L.-I., Grimelius, L., Håkanson, R., Rehfeld, J.F., Stadil, F., Holst, J., Angervall, L., and Sundler, F.: Mixed endocrine pancreatic tumors producing several pep-

tide hormones.

Am.J.Path. 79:271-280, 1975.

36. Larsson, L.-I., Holst, J.J., Kuhl, C., Lundqvist, G., Hitsch, M.A., Ingemansson, S., Lindkaer-Jensen, S., Rehfeld, J.F., and Schwartz, T.W.: Pancreatic somatostatinoma. Clinical features and physiological implications. *Lancet* I:666-668, 1977.
37. Larsson, L.I., Ljungberg, O., Sundler, F., Håkanson, R., Svensson, S.O., Rehfeld, J., Stadil, F., and Holst, J.: Antro-pyloric gastrinoma associated with pancreatic nesidioblastosis and proliferation of islets. *Virchows Arch.Ab.t.A.Path.Anat.* 360:305-314, 1973.
38. Larsson, L.-I., Rehfeld, J.F., Stockbrügger, R., Blohma, G., Schöön, I.-M., Lundqvist, G., Kindblom, L.G., Säve-Söderberg, J., Grimelius, L., and Olbe, L.: Mixed endocrine gastric tumors associated with hypergastrinemia of antral origin. *Am.J.Path.* 93:53-68, 1978.
39. Larsson, L.-I., Sundler, F., and Håkanson, R.: Pancreatic polypeptide. A postulated new hormone: Identification of its cellular storage site by light and electron microscopic immunocytochemistry. *Diabetologia* 12:211-226, 1976.
40. Leichter, S.B.: Clinical and metabolic aspects of glucagonoma. *Medicine* 59:100-113, 1980.
41. Ljungberg, O., Järnerot, G., Rolny, P., and Wickbom, E.: Human pancreatic polypeptide (HPP) immunoreactivity in an infiltrating endocrine tumour of Papilla of Vater with an unusual morphology. *Arch.A.Path.Anat. and Histol.*, (in press), 1981.
42. Martin, E.D., and Potet, F.: Pathology of endocrine tumours of the GI tract. *Clinics Gastroenterol.* 3:511-532, 1974.
43. Marks, C.: Carcinoid tumors. A clinicopathologic study. In G.K. Hall & Co, Boston, pp. 1-154, 1979.
44. Modlin, I.M.: Endocrine tumors of the pancreas. *Surg. Gyn. and Obst.*, 149:752-769, 1979.

45. Morrison, A.B.: Islet cell tumors and the diarrheogenic syndrome.
Internat. Acad.Path.Monogr.Path. 21:155-207, 1980.
46. Pearse, A.G.E., and Polak, J.M.: Bifunctional reagents as vapour- and liquid-phase fixatives for immunohistochemistry.
Histochem.J. 7:179-186, 1975.
47. Rehfeld, J.F.: Heterogeneity of gastrointestinal hormones.
In *Gastrointestinal Hormones*. G.B.J. Glass, ed., Raven Press, New York, pp. 433-449, 1980.
48. Ruttman, E., Klöppel, G., Bommer, G., Kiehn, M., and Heitz, P.U.: Pancreatic glucagonoma with and without syndrome. Immunocytochemical study of 5 tumour cases and review of the literature.
Virchows Arch.A.Path.Anat.and Histol., 388:51-67, 1980.
49. Sherwood, L.M., and Gould, V.E.: Ectopic hormone syndromes and multiple endocrine neoplasia. In *Endocrinology*, eds. de Groot, L.J. et al., vol. 3, Grune & Stratton, New York, pp. 1733-1766, 1979.
50. Soga, J.: Carcinoids: Their changing concepts and a new histologic classification. In *Gastro-Entero-Pancreatic Endocrine System*, ed., T. Fujita, Stuttgart, Thieme, pp. 101-119, 1974.
51. Solcia, S., Capella, C., Buffa, R., Usellini, L., Frigerio, B., and Fontana, P.: Endocrine cells of the gastrointestinal tract and related tumors.
Pathobiol.Ann., 9:163-204, 1979.
52. Sternberger, L.A.: Immunocytochemistry. Ed.2, New York: John Wiley & Sons, pp. 1-354, 1979.
53. Sundler, F., Alumets, J., Ekman, R., Håkanson, R., and van Wimersma Greidanus, T.B.: Immunoreactive ACTH in porcine gut and pancreas. Fact or artefact?
J.Histochem.Cytochem. (in press), 1981.
54. Sundler, F., Alumets, J., Fahrenkrug, J., Håkanson, R., and Schaffalitzky de Muckadell, O.B.: Cellular localization and ontogeny of immunoreactive vasoactive intestinal polypeptide (VIP) in the chicken gut.
Cell Tissue Res. 196:193-201, 1979.

55. Sundler, F., Alumets, J., and Håkanson, R.: 5-Hydroxytryptamine-containing enterochromaffin cells: Storage site of substance P.
Acta Physiol.Scand.Suppl. 451:121-123, 1977.
56. Sundler, F., Alumets, J., and Håkanson, R.: Majority and minority cell populations in GEP and bronchial endocrine tumours.
Scand.J.Gastroent. 14:Suppl. 53:9-13, 1979.
57. Sundler, F., Håkanson, R., Larsson, L.-I., Brodin, E., and Nilsson, G.: Substance P in the gut: An immunohistochemical and immunochemical study of distribution and development. In *Substance P*, U.S. von Euler, and B. Pernow, eds., Raven Press, New York, pp. 55-58, 1977.
58. Vaillant, C., Dockray, G.J., and Hopkins, C.R.: Cellular origins of different forms of gastrin. The specific immunocytochemical localization of related peptides.
J.Histochem.Cytochem. 25:932-935, 1979.
59. Wilander, E.: Achylia, pernicious anaemia. ECL cells and gastric carcinoids.
Virchows Arch.A.Path.Anat. and Histol. 387:371-373, 1980.
60. Wilander, E., Grimelius, L., Lundqvist, G., and Skoog, V.: Polypeptide hormones in argentaffin and argyrophil gastroduodenal endocrine tumors.
Am.J.Path. 96:519-530, 1979.
61. Wilander, E., Grimelius, L., Portela-Gomes, G., Lundqvist, G., Skoog, V., and Westermark, P.: Substance P and enteroglucagon-like immunoreactivity in argentaffin and argyrophil midgut carcinoid tumours.
Scand.J.Gastroent. 14, Suppl. 53, 19-25, 1979.
62. Wilander, E., Portela-Gomes, G., Grimelius, L., and Lundqvist, G.: Enteroglucagon and substance-P-like immunoreactivity in argentaffin and argyrophil rectal carcinoids.
Virchows Arch. B, Cell.Path. 25:117-124, 1977.
63. Wilander, E., Portela-Gomes, G., Grimelius, L., and Westermark, P.: Argentaffin and argyrophil reactions of human gastrointestinal carcinoids.
Gastroenterology 73:733-736, 1977.

64. Woodtli, W., and Hedinger, C.: Inselzelltumoren des Pankreas und ihre Syndrome. Zollinger-Ellison-Syndrom, Glukagonomsyndrom, multiple endokrine Adenomatose und Inselzelltumoren ohne nachweisbare endokrine Aktivität. *Schweiz.Med.Wschr.* 108:1997-2007, 1978.
65. Zollinger, R.M., Ellison, E.C., Fabri, P.J., Johnson, J., Sparks, J., and Carey, L.G.: Primary peptic ulcerations of the jejunum associated with islet-cell tumors. 25 years appraisal. *Ann.Surg.* 192:422-427, 1980.

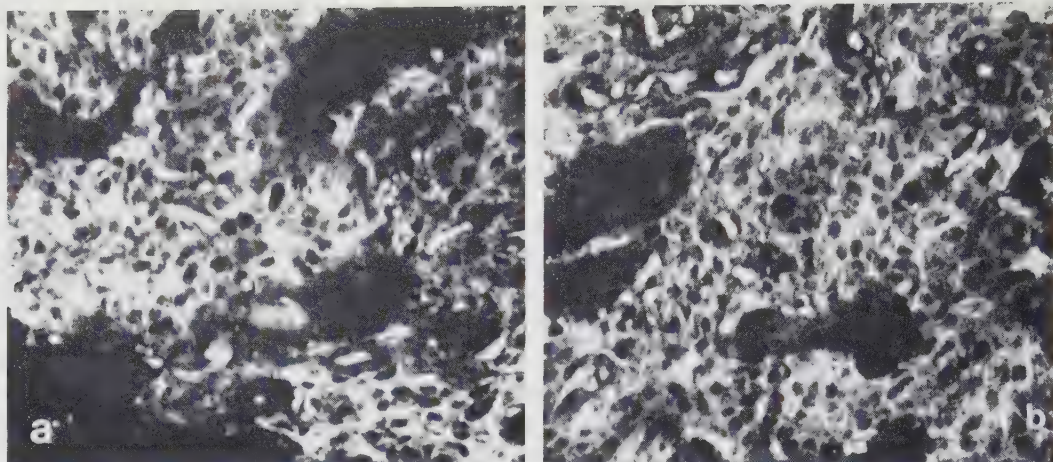


FIGURE 1. Pancreatic tumor (case 2). (a) Insulin immunofluorescence; (b) Calcitonin immunofluorescence. Virtually all tumor cells react with both antisera. (X 200)

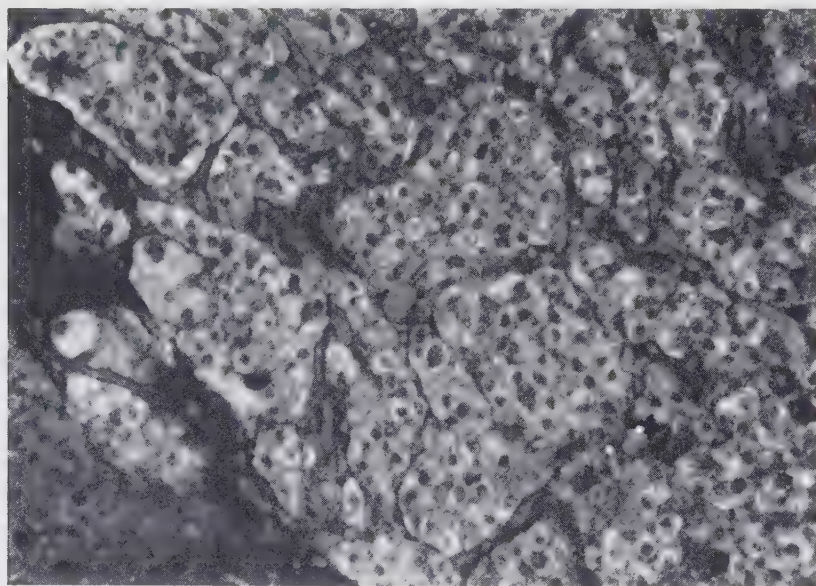


FIGURE 2. Pancreatic tumor (case 5). The bulk of tumor cells display somatostatin immunofluorescence of moderate intensity. In addition, this tumor contains gastrin cells and PP cells as minority cell populations. (X 250)

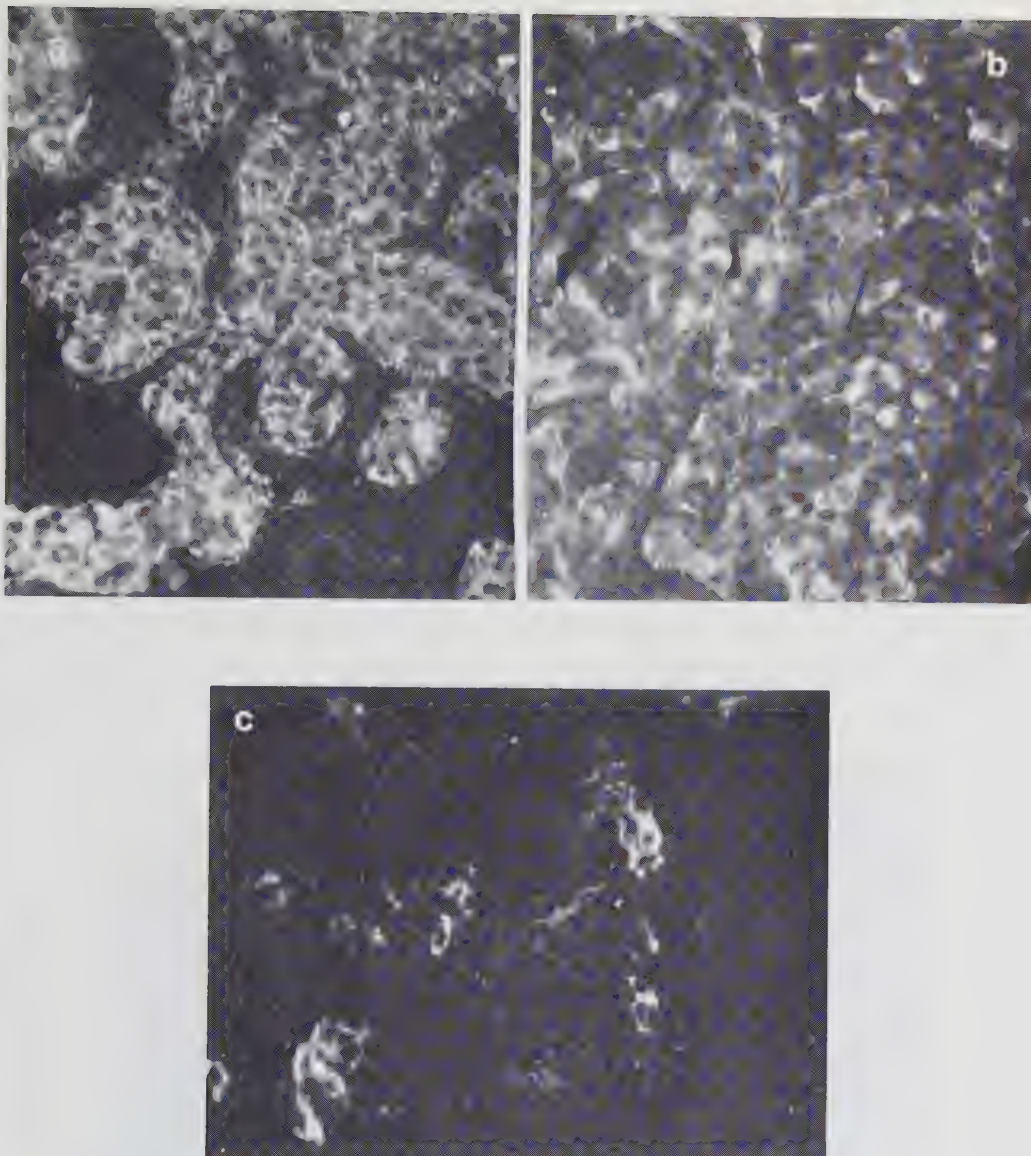


FIGURE 3. Multiple endocrine neoplasia (case 6). Two different pancreatic tumor nodules. One nodule (No. II in Table 3) contains numerous glucagon immunofluorescent cells in one part (a) and PP immunofluorescent cells in another (b). A second nodule (No. III in Table 3) contains insulin cells as a minority cell population (c). This nodule harbors also somatostatin, glucagon, and PP cells as other minority cell populations. (X 200)

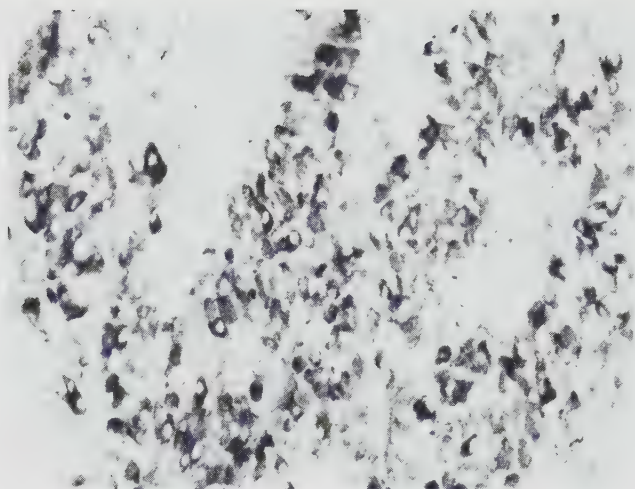


FIGURE 4. Pancreatic tumor nodule from a patient with multiple endocrine neoplasia (case 12), associated with signs of VIP overproduction (the Verner-Morrison syndrome). The bulk of tumor cells display VIP immunoreactivity. PAP staining (X 200)

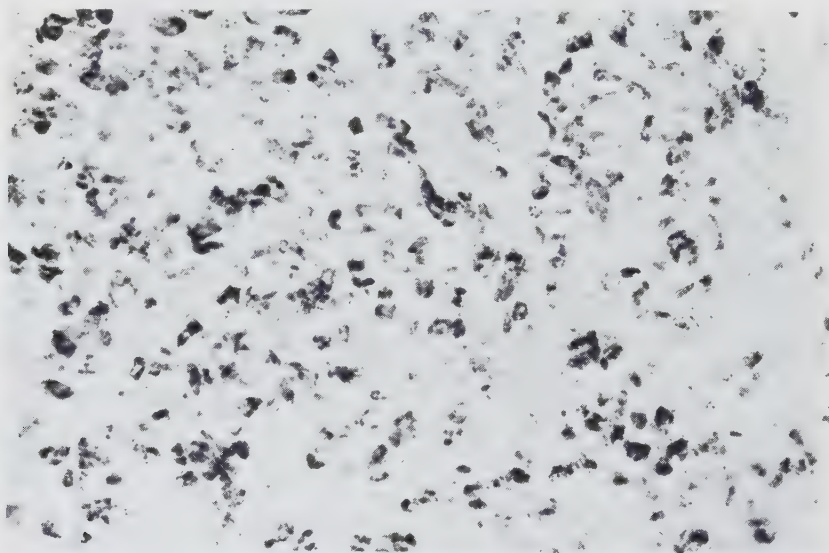


FIGURE 5. Tumor in the corpus region of the stomach (case 16). Numerous cells display gastrin immunoreactivity. The cells could be demonstrated equally well with antisera directed against the COOH terminus of gastrin, the entire gastrin-17 sequence, or against the NH₂ terminus of gastrin-34. The patient did not suffer from signs of gastrin overproduction.

PAP staining (X 200)

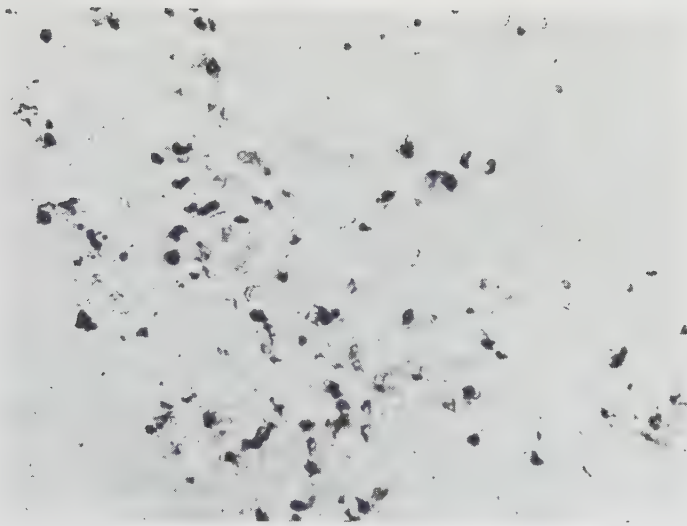


FIGURE 6. Duodenal tumor (case 22), associated with hypergastrinemia and ulcer diathesis. Gastrin immunoreactive cells occur scattered in the tumor parenchyma together with a few somatostatin cells (not shown in figure). PAP staining (X 200)

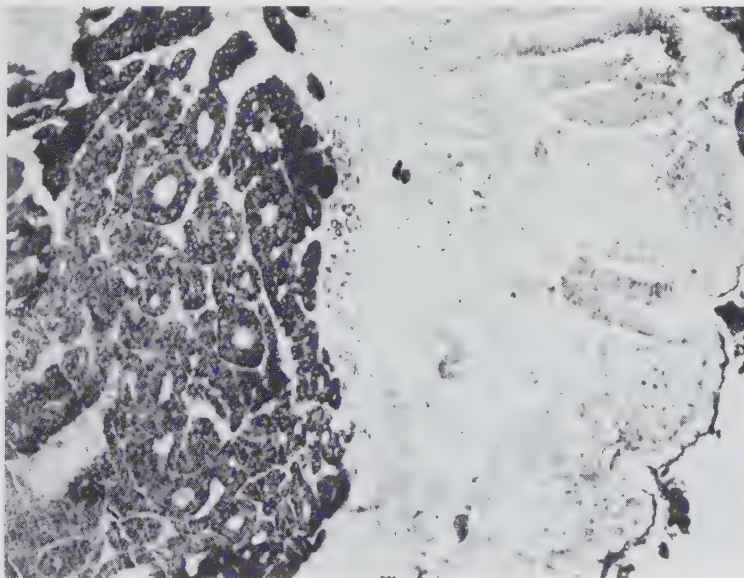


FIGURE 7. Duodenal tumor (case 21). Section through tumor (left) and normal duodenal mucosa (right). Virtually all tumor cells contain immunoreactive somatostatin. This tumor was not associated with any symptoms of endocrine disorder.

PAP staining (X 150)

Substance P and 5-HT in Granules Isolated from an Intestinal Argentaffin Carcinoid

J. Alumets, R. Håkanson, S. Ingemansson, and F. Sundler

Departments of Histology, Pharmacology and Surgery, University of Lund,
Biskopsgatan 5, S-223 62 Lund, Sweden

Summary. Intestinal argentaffin carcinoids, thought to originate from enterochromaffin cells, occasionally contain large amounts of substance P-like immunoreactivity in addition to 5-HT. The cytoplasmic granules of one such tumour were isolated. The granules, which in the electron microscope were shown to be argentaffin, contained both substance P-like immunoreactivity and 5-HT. The results support the view that substance P is localized in a population of enterochromaffin cells where it is stored in the cytoplasmic granules together with 5-HT.

Introduction

The gut is a rich source of substance P which occurs in intrinsic nerves as well as in endocrine-like cells (Nilsson et al., 1975a; Pearse and Polak, 1975). Several observations (recently reviewed by Heitz et al., 1976; Sundler et al., 1977) seem to favour the view that the endocrine-like cells harbouring substance P belong to the enterochromaffin cell system. Particularly pertinent is the presence of substance P-like immunoreactivity in certain intestinal argentaffin carcinoid tumours (Håkanson et al., 1977). These tumours are thought to be derived from the enterochromaffin cell system which is the main site of production and storage of 5-HT. The amine, which is thought to be responsible for the argentaffin and chromaffin reactions (see Vialli, 1966; Vialli and Prenna, 1969; Håkanson et al., 1971), is stored in cytoplasmic granules similar to granules in peptide hormone-secreting cells (cf. Owman et al., 1973). It is a common finding that the granules in such cells store the peptide hormone together with an amine and it seems unlikely that the enterochromaffin cell should represent an exception. In the present study we have isolated the cytoplasmic granules of a carcinoid tumour and shown them to contain both substance P and 5-HT.

Materials and Methods

Case History. Male, 40 years old, with epigastric pains since 1962 was admitted to the Department of Surgery, University Hospital, Lund, in 1976 because of flushing, diarrhea, tachycardia, anemia and greatly elevated urinary 5-HIAA. High peripheral plasma levels of substance P were found

by radioimmunoassay (see Håkanson et al., 1977). Laparotomy revealed multiple ileal tumours and large liver metastases. The tumour was tentatively diagnosed as a carcinoid upon routine histological examination. Ileocecal resection and resection of the left liver lobe were performed leaving a non-resectable large metastasis in the right liver lobe. Four months later hepatic dearterialization was performed. During the latter operation several liver metastases were enucleated; they were subjected to subcellular fractionation or processed for histochemical and ultrastructural analysis as described below.

Material. Antiserum, raised in rabbits against synthetic substance P, was kindly put to our disposal by Dr. G. Nilsson, Department of Pharmacology, Karolinska Institute, Stockholm, Sweden. The antiserum, which has been used in previous immunohistochemical studies (Nilsson et al., 1975a; Håkanson et al., 1977; Sundler et al., 1977) has been characterized by Nilsson et al. (1975b). For control experiments it was inactivated by the addition of excess antigen (10 µg synthetic substance P per ml diluted serum). Fluorescein isothiocyanate-labeled anti-rabbit IgG was purchased from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. It was used in dilution 1:20. Peroxidase-antiperoxidase complex (PAP) was obtained from Cappel Laboratories, Downingtown, Pa., USA and used in dilution 1:80.

Preparation of Tissue. Fresh tumour tissue (9.6 g) was minced carefully and hand-homogenized for a few min with a Potter-Elvehjem type homogenizer (Teflon pestle) in 20 volumes of 0.33 M sucrose. These and subsequent manipulations were carried out at 0° C. The homogenate was centrifuged at 1000 × g for 20 min in a refrigerated centrifuge (Sorvall Superspeed RC 2-B) and the supernatant was then passed through a series of MF Millipore® filters (47 mm in diameter) with diminishing pore size: 8000, 5000, 3000, 1200 and 800 nm. The filtrate was divided into three equal portions, which were centrifuged at 40,000 × g for 30 min. One pellet was used for determination of 5-HT (see below). The second pellet and small pieces of the tumour were fixed in a few ml of 2.5% glutaraldehyde in 0.075 M phosphate buffer (Sörensen), pH 7.2 (for electron microscopy). Most of the specimens were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol solutions, contrasted in a mixture of 1% phosphotungstic acid and 0.5% uranyl acetate and embedded in Epon. Other pellet fragments were processed in the same way but without osmium tetroxide. Ultrathin sections were cut on an LKB Ultratome, contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope. Other tumour specimens and the third pellet were frozen to the temperature of liquid nitrogen in a mixture of propane and propylene and freeze-dried (for fluorescence and light microscopy). The freeze-dried material was fragmented and the various fragments were exposed either to formaldehyde gas (Björklund et al., 1972) or diethylpyrocarbonate vapour (Pearse et al., 1974) and embedded in paraffin.

Determination of 5-HT. The pellet was blotted dry, weighed and transferred to 5 ml of 80% aqueous acetone. After 24 h at +4° C the acetone extract was evaporated to dryness *in vacuo*, the dry residue was dissolved in 1 ml of 0.1 N HCl, made alkaline with 0.5 ml of 10% Na₂CO₃ and extracted with 15 ml of n-butanol. 5-HT was then returned to an aqueous phase by shaking with 2 ml 0.1 N HCl and determined fluorometrically after condensation with OPT according to Maickel et al. (1968).

Histochemistry. Both tumour tissue and pellets were analyzed. Paraffin sections were cut at 5 µ. The formaldehyde-fixed specimens were mounted in Entellan and examined for formaldehyde-induced fluorescence. The diethylpyrocarbonate-fixed specimens were subjected to the indirect immunofluorescence procedure (Coons, Leduc and Conolly, 1955) or the PAP procedure (Sternberger, 1974) for the demonstration of substance P-like immunoreactivity. The substance P antiserum was used in dilution 1:20 (immunofluorescence) or 1:80 (PAP staining).

In one series of experiments, sections from the diethylpyrocarbonate-fixed granule pellet were stained with the PAP procedure using active or inactivated anti-substance P serum. The staining intensity was evaluated semi-quantitatively in the following manner: Arbitrarily selected visual fields were photographed at magnification 125 X (12.5 X eye-piece, 10 X objective) with a photo-cell-connected automatic camera. The time of exposure, which is proportional to the staining intensity, was recorded.

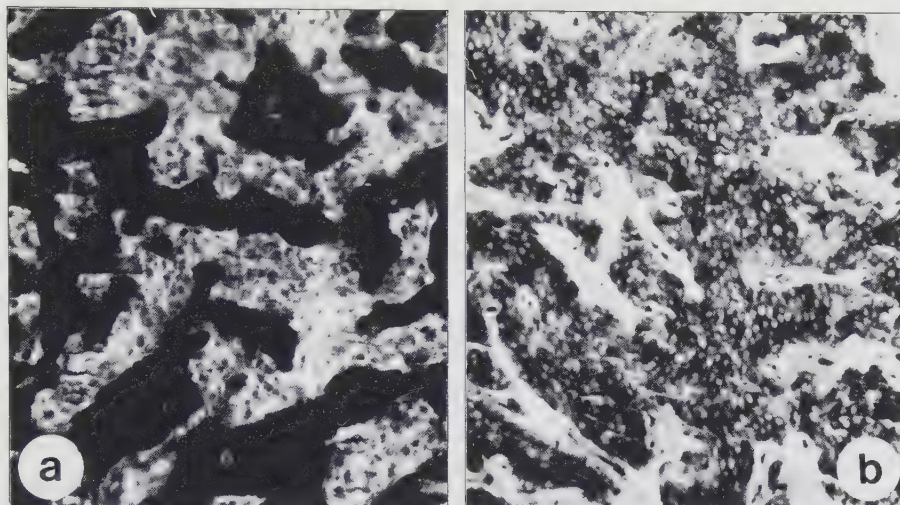


Fig. 1a and b. Carcinoid tumour. **a** Formaldehyde vapour fixation. The bulk of tumour cells exhibit intense 5-HT fluorescence. **b** Diethylpyrocarbonate fixation. Immunohistochemical staining (*PAP*) of substance P. $\times 150$

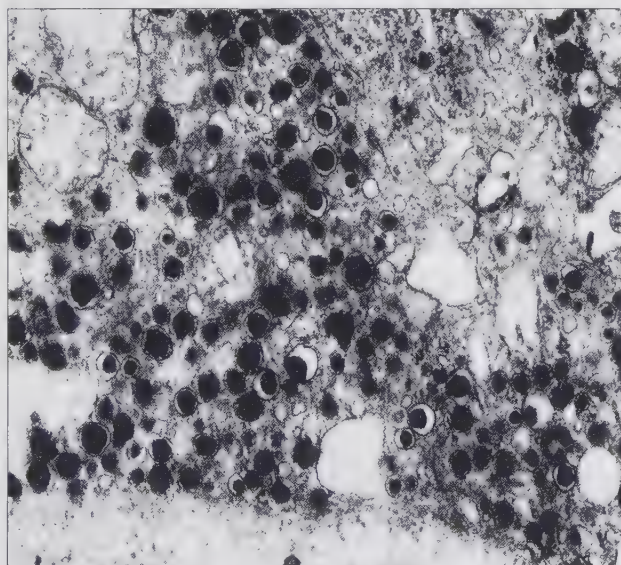


Fig. 2. Electron micrograph of carcinoid tumour cell. Formaldehyde-glutaraldehyde-osmium tetroxide fixation. Numerous pleiomorphic, highly electron-dense cytoplasmic granules. $\times 23,500$

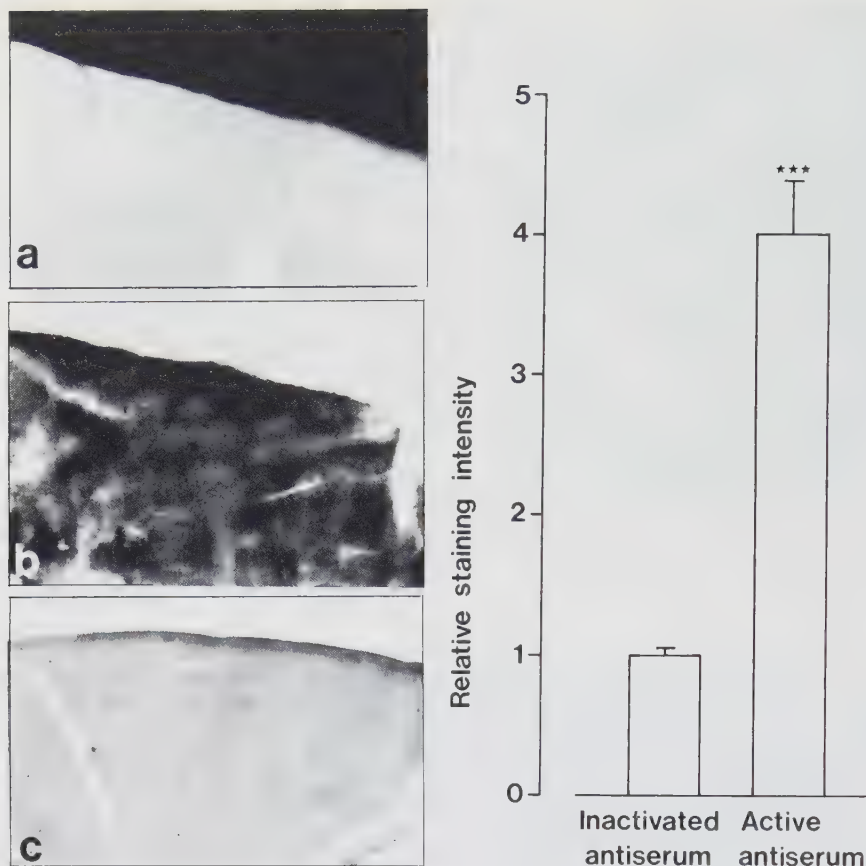


Fig. 3a–c. Granular pellet. **a** Formaldehyde vapour fixation. Intense 5-HT fluorescence. **b** and **c** Diethylpyrocarbonate fixation. PAP-staining for substance P using active anti-substance P serum (**b**) and inactivated antiserum (**c**) ($\times 150$). Panel showing intensity of PAP stain (arbitrary units) in sections from the granular pellet using active and inactivated antiserum, respectively. Each value is the mean of 10 separate recordings. Vertical bars give S.E.M. The difference is significant by $p < 0.005$ (Student's *t*-test). For details see text

Paraffin sections from tumour material and ultrathin sections from non-osmicated pellet fragments (placed on nickel grids) were stained with ammoniacal silver nitrate in the argentaffin procedure of Singh (1964) (cf. Håkanson et al., 1971).

Results and Comments

The bulk of tumour cells displayed substance P-like immunoreactivity of varying intensity and moderate to intense formaldehyde-induced 5-HT fluorescence (Fig. 1). As could be expected most of the tumour cells proved argentaffin. In the electron microscope, the tumour cells were characterized by the presence

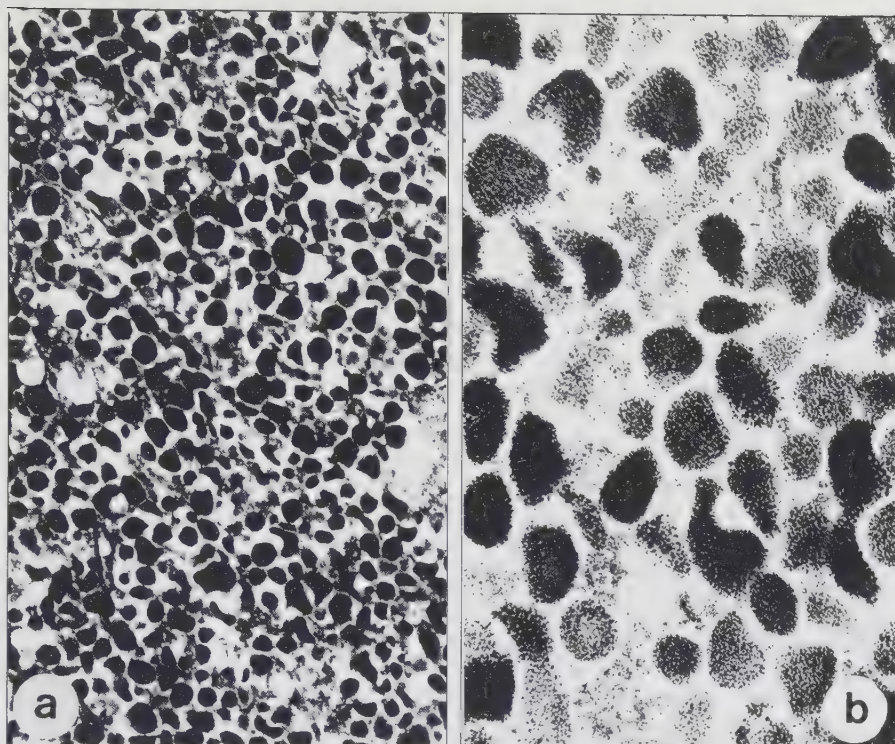


Fig. 4a and b. Granular pellet. Formaldehyde-glutaraldehyde fixation. No osmium tetroxide. **a** Conventional section-contrasting with lead citrate and uranyl acetate. Densely packed, highly electron-dense, pleiomorphic granules. $\times 20,000$. **b** Argentaffin reaction. Silver precipitate over granules. Note varying staining intensity. No contrasting. $\times 63,500$

of electron-dense, pleiomorphic cytoplasmic granules (Fig. 2). The granular pellet was rich in 5-HT (more than $120 \mu\text{g}$ per gram wet weight). Upon histochemical examination it was found to be homogenous, exhibiting intense formaldehyde-induced 5-HT fluorescence and moderate to strong substance P-like immunoreactivity (Fig. 3). In the electron microscope the pellet was found to consist almost exclusively of membrane-bound, pleiomorphic granules (Fig. 4a) with the appearance of enterochromaffin cell granules. All of them displayed argentaffin staining of varying intensity (Fig. 4b). We therefore conclude that both 5-HT and substance P are contained in the cytoplasmic granules of the carcinoid tumour cells. The findings of the present study support the view that substance P is produced, stored and secreted by a certain type of enterochromaffin cell and that the peptide occurs together with 5-HT in the cytoplasmic granules.

Acknowledgments. Grant support from the Swedish Medical Research Council (04X-1007, 04X-4499) and Riksföreningen mot Cancer (806-02X).

References

- Björklund, A., Falck, B., Owman, Ch.: Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic monoamines. In: *Methods of investigative and diagnostic endocrinology*, vol. 1 (J.E. Rall and I.J. Kopin, eds) pp. 318–368. Amsterdam: North-Holland 1972
- Coons, A.H., Leduc, E.H., Conolly, J.M.: Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* **102**, 49–60 (1955).
- Håkanson, R., Bengmark, S., Brodin, E., Ingemansson, S., Larsson, L.-I., Nilsson, G., Sundler, F.: Substance P-like immunoreactivity in intestinal carcinoid tumours. Nobel Symp. on Substance P, Stockholm 1976, to be published 1977
- Håkanson, R., Owman, Ch., Spörng, B., Sundler, F.: Electron microscopic classification of amine-producing endocrine cells by selective staining of ultra-thin sections. *Histochemie* **27**, 226–242 (1971)
- Heitz, Ph., Polak, J.M., Timson, C.M., Pearse, A.G.E.: Enterochromaffin cells as the endocrine source of gastrointestinal substance P. *Histochemistry* **49**, 343–348 (1976)
- Maickel, R.P., Cox, R.H., Saillant, J., Miller, F.P.: A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. *Int. J. Neuropharmacol.* **7**, 275–281 (1968)
- Nilsson, G., Larsson, L.-I., Håkanson, R., Brodin, E., Pernow, E., Sundler, F.: Localization of substance P-like immunoreactivity in mouse gut. *Histochemistry* **43**, 97–99 (1975a)
- Nilsson, G., Pernow, B., Fischer, G.H., Folkers, K.: Presence of substance P-like immunoreactivity in plasma from man and dog. *Acta physiol. scand.* **94**, 542–544 (1975b)
- Owman, Ch., Håkanson, R., Sundler, F.: Occurrence and function of amines in endocrine cells producing polypeptide hormones. *Fed. Proc.* **32**, 1785–1791 (1973)
- Pearse, A.G.E., Polak, J.M.: Immunocytochemical localization of substance P in mammalian intestine. *Histochemistry* **41**, 373–375 (1975)
- Pearse, A.G.E., Polak, J.M., Adams, C., Kendall, P.A.: Diethylpyrocarbonate, a vapour-phase fixative for immunofluorescence studies on polypeptide hormones. *Histochem. J.* **6**, 347–352 (1974)
- Singh, I.: A modification of the Masson-Hamperl method for staining of argentaffin cells. *Anat. Anz.* **115**, 81–82 (1964)
- Sternberger, L.A.: *Immunocytochemistry*, p. 129. New Jersey: Prentice-Hall, Inc. 1974
- Sundler, F., Håkanson, R., Larsson, L.-I., Brodin, E., Nilsson, G.: Substance P in the gut: An immunochemical and immunohistochemical study of its distribution and development. Nobel Symp. on Substance P, Stockholm 1976, to be published 1977
- Vialli, M., Prenna, G.: Contribution to the cytospectrofluorometric measurement of 5-hydroxytryptamine in enterochromaffin cells. *J. Histochem. Cytochem.* **15**, 321–330 (1969)
- Vialli, M.: Histology of the enterochromaffin cell system. In: *Handbook of Experimental Pharmacology*. Vol. XIX, 5-Hydroxytryptamine and related indolealkylamines, pp. 1–65. Berlin, Heidelberg, New York: Springer 1966

Received February 10, 1977

IMMUNOHISTOCHEMICAL EVIDENCE OF PEPTIDE HORMONES IN ENDOCRINE TUMORS OF THE RECTUM

J. Alumets*, M.D., P. Alm**, M.D., Ph.D., S. Falkmer****, M.D., Ph.D.,
R. Håkanson***, M.D., Ph.D., O. Ljungberg*****, M.D., Ph.D., H. Mårten-
sson****, M.D., F. Sundler*, M.D., Ph.D. and S. Tibblin*****, M.D., Ph.D.

From the Departments of Histology*, Pathology**, Pharmacology*** and Surgery****,
University of Lund, Lund and the Departments of Pathology***** and Surgery*****,
University of Lund at Malmö General Hospital, Malmö, Sweden

Abstract. Twentyfive endocrine tumors of the rectum (rectal carcinoids) were examined immunohistochemically for various pancreatic and gut neurohormonal polypeptides. Twentyone of the tumors were found to contain cells displaying pancreatic polypeptide (PP), glucagon, somatostatin, insulin, substance P, enkephalin or β -endorphin immunoreactivity. At least eleven of the tumors contained more than one peptide hormone. In some of the tumors PP cells made up the major cell population, in others the glucagon cells. Only three of the tumors contained 5-HT. Rectal endocrine tumors seem unique among gut endocrine tumors in that they may store immunoreactive enkephalin, β -endorphin and even insulin. None of the patients displayed the carcinoid syndrome; symptoms were usually vague and uncharacteristic. In many cases the tumor was found at routine examination.

The number of recognized neurohormonal peptides in the gut has increased greatly during recent years¹⁴. As a consequence, peptide hormone production in endocrine tumors of the gut has attracted growing interest. Tumors giving rise to spectacular and specific constellations of clinical symptoms such as the Zollinger-Ellison, the watery diarrhea (WDHA), the insulinoma and the glucagonoma syndromes^{16,20,29} are usually located in the pancreas¹⁷. Those causing the carcinoid syndrome are usually located in the lower small intestine⁸. So far, immunohistochemical studies in search of peptides in endocrine tumors have been focused on those with a foregut or midgut origin^{2,6,17,18,31}. Hindgut carcinoids are as a rule "silent" in that they rarely give rise to spectacular symptoms of endocrine disorders^{22,26,28}. They are often found incidentally at routine examination or at autopsy²⁸. Nevertheless, it cannot be excluded that also hindgut carcinoids may produce neurohormonal peptides. Thus, there is evidence for the presence of immunoreactive substance P, enteroglucagon or endogenous opioid peptides in rectal carcinoids^{1,32}. We have now examined 25 rectal carcinoids* by immunohistochemistry, using antisera raised against various pancreatic and gut neurohormonal peptides.

MATERIAL AND METHODS

Biopsy and/or operation specimens from 25 rectal carcinoids were examined. They were routinely prepared for histopathological examination (fixation with 10% neutral formalin, dehydration, paraffin embedding and sectioning at 5 μ m). For conventional light microscopic analysis the sections were stained with van Gieson's stain, Weigert's elastin, hematoxylin-eosin, periodic-acid-Schiff (PAS) reagent, or with silver nitrate according to the Masson-Hamperl argentaffin²⁵ or the Grimelius argyrophil¹³ procedures.

For immunocytochemical analysis deparaffinized sections were thoroughly rinsed in 0.1 M phosphate buffer, pH 7.2, for 12-24 hrs and then subjected to the indirect immunofluorescence method⁷, or the peroxidase-anti-peroxidase (PAP) technique³⁰. Details on the antisera giving immunostaining of the tumors are given in Table 1. For controls we used sections exposed to antiserum inactivated by the addition of antigen

*Footnote: The word "carcinoid" is used here to signify gut endocrine tumors in general.

in excess (10–100 $\mu\text{g}/\text{ml}$ diluted antiserum). Details of the immunocytochemical protocol used are given elsewhere^{3,4}. 5-Hydroxytryptamine (5-HT) was demonstrated by its characteristic yellow formaldehyde-induced fluorescence upon excitation at 365 nm².

RESULTS

Sixteen of the 25 patients were women. The age distribution was broad with 5 patients under 40 years of age. The tumors, which were less than 2 cm in diameter in all cases but one (No. 3) were always solitary and found in the rectal ampulla within 10 cm above the anus. A regional lymph node metastasis was found in one patient only (No. 21). Symptoms, when present, were vague. In 11 patients the rectal tumor was found incidentally or at health screening. Seven of the remaining 17 patients suffered from rectal bleeding, 8 complained of obstipation and/or diarrhea, and 2 reported pain in the sacral and anal region.

No patients exhibited symptoms of the carcinoid syndrome or other specific constellations of endocrine symptoms at the time of operation or during the subsequent follow-up period (two to twelve years). Three patients suffered from neoplastic diseases unrelated to their endocrine tumor. For a summary see Table 2.

The tumors were classified according to their growth pattern^{27,28} (Table 2). Most tumors displayed a trabecular or ribbon-like structure (Fig. 1).

Nineteen of the tumors were found to contain cells staining with one or more of the antisera used (Table 3). Thus, pancreatic polypeptide (PP) immunoreactive cells were found in 14 of the tumors; in 9 of the tumors PP cells constituted the major cell population (Fig. 2a). Glucagon immunoreactive cells were found in 12 tumors; they made up the major cell population in 8 (Fig. 2b). Six tumors contained somatostatin immunoreactive cells; the cells were invariably few or moderate in number (Fig. 2c). Enkephalin immunoreactive cells were found only in those two tumors previously described¹. In one of these tumors the enkephalin cells made up the major cell population (Fig. 2d) while in the other the cells were scarce. The latter tumor, in addition, harboured scattered cells displaying β -endorphin immunoreactivity. Insulin immunoreactive cells were found in two tumors, constituting the major cell population in both of them (Fig. 3a). One of these tumors contained a wide variety of immunoreactive peptides as well as 5-HT. Three tumors contained 5-HT-storing cells, which also were argentaffin. Only in one of

these tumours could substance P immunoreactive cells be detected (Fig. 3b).

Eleven of the tumors contained more than one identified peptide-storing cell population; ten tumors contained only one identifiable cell population; four tumors were non-reactive to all antisera tested. None of the tumors contained cells reacting with antisera raised against ACTH, gastrin/CCK, GIP, motilin, neurotensin, secretin or VIP. These antisera and the reaction conditions have been described elsewhere¹.

DISCUSSION

Endocrine tumors of the gut and pancreas are often classified according to their histological growth pattern^{8,27,28}; tumors arising in the foregut, midgut and hindgut differ from each other in this respect. In addition, various histological staining techniques, notably the silver staining procedures of Masson-Hamperl²⁵ and Grimelius¹³ have been used to aid in the classification of such tumors. Also the clinical symptoms are of importance for the classification. A well-known example is the carcinoid syndrome, which is associated with 5-HT-producing argentaffin tumors^{2,15} usually arising in the small intestine. By contrast, the hindgut carcinoids presented here are typical examples of silent tumors. Generally, the uneventful postoperative course and low tendency to local and distant spread found in our cases support previous reports that hindgut carcinoids, when small (< 2 cm), generally have a benign clinical course^{8,12,26,28}.

The majority of the hindgut tumors examined displayed a trabecular or ribbon-like growth pattern^{26,28}. There was no correlation between the growth pattern and the presence or absence of a given peptide; the same applies to argyrophilia, argentaffinity, and presence or absence of 5-HT (Table 3).

The variety of peptide hormones and hormone candidates detected by immunocytochemistry in the rectal tumors examined was remarkable. Not surprisingly^{17,31}, many of the tumors were heterogenous in that they contained several distinct peptide-producing cell types. In this respect they resembled tumors of foregut origin¹⁷. The immunoreactive peptides observed in the rectal tumors included PP, glucagon, somatostatin, insulin, substance P, enkephalin and β -endorphin. Many of these peptides are normally associated primarily with the stomach, small intestines and pancreas; only PP-, glucagon- and somatostatin-like immunoreactivity has been found in endocrine cells of the normal large intestine^{4,5}.

PP has previously been found to be a fairly common constituent of pancreatic endocrine

tumors²³. PP has been put forward as a causative agent in the WDHA syndrome^{18,19} although vasoactive intestinal peptide (VIP) is generally thought to be the major responsible factor^{9,20,34}. In spite of the fact that PP cells made up the majority cell population in several of the present rectal tumors, the patients did not suffer from diarrhoea. Although glucagon immunoreactive cells were detected in 12 tumors and insulin immunoreactive cells in 2 tumors, no disturbances in the glucose metabolism were observed in these patients. It is probable that immunoreactive glucagon in rectal tumors, as in the supposed parent cells in the normal rectal mucosa, represents gut type glucagon (glicentin)^{21,24}, which does not seem to have the same spectrum of biological activity as pancreatic type glucagon. Nevertheless, the conspicuous lack of clinical symptoms suggests that peptides in rectal carcinoids may be secreted in an inactive form, not at all secreted, or rapidly inactivated in the circulation.

The occurrence of immunoreactive insulin in the rectal carcinoids is noteworthy since insulin in its phylogenetically earliest form occurs in cells of the gut epithelium¹¹.

It must be emphasized that demonstration of immunoreactivity does not prove the existence of the antigen in the cells. The immunoreactivity may reflect the presence of cross-reacting peptides similar to but distinct from the authentic hormone.

In some of the tumors only one peptide hormone-containing cell population could be detected and in a few cases no immunoreactive cells were found with the antisera used. It is to be expected that reexamination of such tumors using antisera against other peptides should reveal additional immunoreactive cell populations.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Cancer Society (Project No. 806-05xA), The Swedish Medical Research Council (Project Nos. 04x4499 and 12x718) and the Cancer Research Foundation at Malmö General Hospital.

A preliminary report¹⁰ was given at the Hiroshima Symposium on Gut Hormones, July 17-19, 1979.

Correspondence to: Dr. Jan Alumets, Dept. of Histology, Biskopsgatan 5, S-22362 Lund, Sweden.

REFERENCES

1. Alumets, J., Falkmer, S., Grimelius, L., Håkanson, R., Ljungberg, O., Sundler, F., and Wilander, E.: Immunocytochemical demonstration of enkephalin and β -endorphin in endocrine tumors of the rectum. A survey of 27 colo-rectal carcinoids. Acta path.microbiol.scand. Sect. A, 88: 103-109, 1980.
2. Alumets, J., Håkanson, R., Ingemansson, S., and Sundler, F.: Substance P and 5-HT in granules isolated from an intestinal argentaffin carcinoid. Histochemistry 52: 217-222, 1977.
3. Alumets, J., Håkanson, R., Sundler, F., and Chang, K.-J.: Leu-enkephalin-like material in nerves and enterochromaffin cells in the gut. Histochemistry 56: 187-196, 1978.
4. Alumets, J., Sundler, F., and Håkanson, R.: Distribution, ontogeny and ultrastructure of somatostatin immunoreactive cells in the pancreas and gut. Cell Tiss. Res. 185: 465-479, 1977.
5. Buffa, R., Capella, C., Fontana, P., Usellini, L., and Solcia, E.: Types of endocrine cells in the human colon and rectum. Cell Tiss. Res. 192: 227-240, 1978.
6. Capella, C., Solcia, E., Frigerio, B., Buffa, R., Usellini, L., and Fontana, P.: The endocrine cells of the pancreas and related tumours. Virchows Arch.A.Path. Anat. and Histol. 373: 327-352, 1977.
7. Coons, A.H., Leduc, E.H., and Conolly, J.M.: Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. 102: 49-60, 1955.
8. Dawson, I.M.P.: The endocrine cells of the gastrointestinal tract and the neoplasms which arise from them. Curr. Topics Path. 63: 221-259, 1976.
9. Ebeid, A.M., Murray, P.D., and Fischer, J.E.: Vasoactive intestinal peptide and the watery diarrhea syndrome. Ann. Surg. 187: 412-416, 1978.
10. Falkmer, S., Alumets, J., Håkanson, R., Ljungberg, O., Sundler, F., and Tibblin, S.: Occurrence of pancreatic polypeptide (PP), somatostatin, glucagon, insulin, enkephalin, β -endorphin, and substance P cells in rectal carcinoids. A preliminary report of nineteen cases. In: Gut Peptides, Secretion, Function and Clinical Aspects. A. Miyoshi, ed. Amsterdam: Elsevier/North-Holland, 1979, pp. 351-355.

11. Falkmer, S., Östberg, Y.: Comparative morphology of pancreatic islets in animals. In: The Diabetic Pancreas, B.V. Volk and K.F. Wellman, Eds. Plenum Publ., New York, 1977; pp. 15-59.
12. Genre, C.F., Roth, L.M., and Reed, R.J.: "Benign" rectal carcinoids: A report of two patients with metastases to regional lymph nodes. Am.J.Clin.Pathol. 56: 750-757, 1971.
13. Grimelius, L.: A silver nitrate stain for A₂-cells in human pancreatic islets. Acta Soc. Med.Upsalien. 73: 243-270, 1968.
14. Håkanson, R., and Sundler, F.: Peptidergic system of the gastrointestinal tract. Verhandl.Dtsch.Ges.Inn.Med. 85: 1525-1534, 1979.
15. Håkanson, R., Bengmark, S., Brodin, E., Ingemansson, S., Larsson, L.-I., Nilsson, G., and Sundler, F.: Substance P-like immunoreactivity in intestinal carcinoid tumors. In: Substance P, U.S. von Euler, and B. Pernow, Eds. Raven Press, New York, 1977; pp. 55-58.
16. Holst, J.J.: Gut endocrine tumour syndromes. Clinics Endocr. Metab. 8: 413-432, 1979.
17. Larsson, L.-I., Grimelius, L., Håkanson, R., Rehfeld, J.F., Stadil, F., Holst, J., Angervall, L., and Sundler, F.: Mixed endocrine pancreatic tumours producing several peptide hormones. Am.J.Pathol. 79: 271-285, 1975.
18. Larsson, L.-I., Schwartz, T., Lundquist, G., Chance, R.E., Sundler, F., Rehfeld, J.F., Grimelius, L., Fahrenkrug, J., Schaffalitzky de Muckadell, O., and Moon, N.: Occurrence of human pancreatic polypeptide in pancreatic endocrine tumors. Am.J.Pathol. 85: 675-684, 1976.
19. Lundquist, G., Krause, U., Larsson, L.-I., Grimelius, L., Schaffalitzky de Muckadell, O.B., Fahrenkrug, J., Johnson, M., and Chance, R.E.: A pancreatic-polypeptide-producing tumour associated with the WDHA syndrome. Scand. J. Gastroent. 13: 715-718, 1978.
20. Modlin, I.M., Bloom, S.R., and Mitchell, S.: VIP: The cause of the watery diarrhoea syndrome. Adv. Exp.Med.Biol. 106: 195-201, 1978.
21. Moody, A.J., Jacobsen, H., and Sundby, F.: Gastric glucagon and gut glucagon-like immunoreactants. In: Gut Hormones, S.R. Bloom, Ed., Churchill-Livingstone, London, 1978; pp. 369-378.

22. Orloff, M.J.: Carcinoid tumors of the rectum. Cancer 28: 175-180, 1971.
23. Polak, J.M., Adrian, T.E., Bryant, M.G., Bloom, S.R., Heitz, P., and Pearse, A.G.E.: Pancreatic polypeptide in insulinomas, gastrinomas, vipomas, and glucagonomas. Lancet I: 328-330, 1976.
24. Ravazzola, M., Siperstein, A., Moody, A.J., Sundby, F., Jacobsen, H., and Orci, L.: Glicentin immunoreactive cells: Their relationship to glucagon-producing cells. Endocrinology 105: 499-508, 1979.
25. Romeis, B.: Mikroskopische Technik 15, R. Oldenbourg, Ed. München, 1978; p. 164.
26. Rumpf, P., Ulrich, B., Krian, A., and Borchard, F.: Rektumkarzinoide. Dtsch.med.Wschr. 101: 1531-1533, 1976.
27. Soga, J., and Tazawa, K.: Pathologic analysis of carcinoids. Histologic reevaluation of 62 cases. Cancer 28: 990-998, 1971.
28. Soga, J.: Carcinoids: Their changing concepts and a new histologic classification. In: Gastro-Entero-Pancreatic Endocrine System, T. Fujita, Ed. Georg Thieme Publ., Stuttgart, 1974; pp. 101-119.
29. Stadil, F., and Stage, J.G.: The Zollinger-Ellison Syndrome. Clinics Endocr. Metab. 8: 433-446, 1979.
30. Sternberger, L.A.: Immunocytochemistry. Prentice Hall, Inc. Englewood Cliffs, New Jersey, 1974.
31. Sundler, F., Alumets, J., and Håkanson, R.: Majority and minority cell populations in GEP and bronchial endocrine tumours. Scand.J.Gastroent. 14, Suppl. 53: 9-13, 1979.
32. Wilander, E., Portela-Gomes, G., Grimelius, L., Lundquist, G., and Skoog, V.: Enteroglucagon and substance P-like immunoreactivity in argentaffin and argyrophil rectal carcinoids. Virchows Arch.B.Cell Path. 25: 117-124, 1977.
33. Wilander, E., Portela-Gomes, G., Grimelius, L., and Westermarck, P.: Argentaffin and argyrophil reactions of human gastrointestinal carcinoids. Gastroenterology 73: 733-736, 1977.
34. Wu, Z.C., O'Dorisio, T.M., Cataland, S., Mekhjian, H.S., and Gaginella, T.S.: Effects of pancreatic polypeptide and vasoactive intestinal polypeptide on rat ileal and colonic water and electrolyte transport in vivo. Dig.Dis.Sci 24: 625-630, 1979.

Source and working dilutions of antisera giving immunostaining of the endocrine rectal tumors

Antisera against	Antigen	Immu- fluorescence staining	Working dilution PAP	Code	Source
β -Endorphin	Synthetic human β -endorphin (β -lipotropin 61-91)	1:20	1:640	7762	Å. Forsberg, Milab, Malmö, Sweden
Enkephalin	Synthetic leu-enkephalin	1:60	1:240	"leu-enk"	R. J. Miller and K.-J. Chang, Wellcome Res. Lab., Burroughs Wellcome Co., Triangle Park, N.C., USA
Glucagon	Pure porcine pancreatic glucagon**	1:20	1:2560	4304*	J. Holst, Dept. Clin. Chem., Bispebjerg Hospital, Copenhagen, Denmark
Insulin	Pure bovine insulin**	1:80	1:5120	LAI	L. Heding, Novo Res. Inst., Copenhagen, Denmark
Pancreatic Polypeptide (PP)	1) Pure human PP** 2) Pure bovine PP**	1) 1:20 2) 1:80	— 1:2560	HPP BPP	1) and 2) R. Chance, Eli Lilly Indianapolis, Ind., USA
Somatostatin	Synthetic ovine somatostatin	1:40	1:1280	19578	M. P. Dubois, Inst. Nat. Rech. Agr., Nouzilly, France
Substance P	Synthetic bovine substance P	1) 1:20 1:20	— 1:160	K25 K16	1) G. Nilsson, Dept. Pharmacol., Karolinska Institute, Stockholm, Sweden
		2) 1:320 1:20	1:640 1:160	JK1 SP8	

* This antiserum cross-reacts with gut-type glucagon (glucintin)

** The following references give details on the purification of the antigen and the production of antisera

glucagon: Holst, J. J., and Aasted, B.: Production and evaluation of glucagon antibodies for radioimmunoassay. Acta endocrinol. (Kbh) 77: 715-726, 1974insulin: Heding, L. G.: Determination of total serum insulin (IRI) in insulin-treated diabetic patients. Diabetologia 8: 260-266, 1972
PP: Larsson, L.-I., Schwartz, T., Lundqvist, G., Chance, R. E., Sundler, F., Rehfeld, J. F., Grimelius, L., Fahrenkrug, J., Schaffalitzky de Muckadell, O., and Moon, N.: Occurrence of human pancreatic endocrine tumors. Possible implication in the watery diarrhea syndrome. Am. J. Pathol. 85: 675-684, 1976

TABLE 2

Review of the 20 patients with endocrine tumors of the rectum

No	Age	Patient Code (No/year)	Sex	Histol type ^{a)}	Argyrophil ^{b)}	Argentaffin ^{c)}	5-HT	Other tumors
1	53	8329/72	M	E	+	+	+	pineal
2	55	2134/75 2670/75	F	B	-	-	-	
3	53	3017/78	F	D	+	-	-	
4	42	15093/79	F	B	-	-	-	
5	42	6093/71	F	A	-	-	-	
6	22	5362/70	F	B	-	-	-	
7	56	6006/79	M	A	-	+	-	
8	44	3527/68	F	B	-	-	-	
9	77	8829/74	F	C	-	-	-	colon
10	34	4254/80	M	E	-	-	-	
11	40	19049/71	M	B	-	-	-	
12	55	2609/69	M	E	-	-	-	
13	35	7923/70	F	B	+	-	-	
14	59	21508/76	F	B	-	-	-	
15	56	18608/69	M	E	+	+	+	urinary bladder
16	61	18693/69	F	E	-	-	-	
17	51	337/77	F	B	+	-	-	
18	68	7911/70	M	A	-	-	-	
19	80	14588/77	M	C	+	+	+	
20	54	9149/65	F	B	+	-	-	
21	49	776/77	F	B	+	-	-	
22	28	15011/78	F	B	+	-	-	
23	29	14260/78	F	B	-	-	-	
24	60	1286/61	F	A	-	-	-	
25	67	8663/71	M	A	-	-	-	

a) according to Soga;^{27,28} A = "solid"; B = "trabecular"; C = "tubular"; D = "undifferent"; E = "mixed types"

b) Grimelius technique¹³ c) Masson Hamperl technique²⁵

+ and - denote presence and absence, respectively, of argyrophil or argentaffin material or 5-HT in the tumor cells

TABLE 3

Immunohistochemical features of the tumors^{a)}

No	Pancreatic polypeptide (PP)	Glucagon	Somato- statin	Insulin	Substance P	Enkephalin	β-Endorphin
1	++	++	++	+++	++		
2	+++	+	+	+++			
3	++	++				++	
4	++	+++					
5	+	++					
6	+++	++					
7	+	+++					
8	+		+				
9	+++						
10	+++						
11	++						
12	++						
13	+						
14	+						
15		+	+				
16		+	+				
17		+++					
18		+++					
19		+					
20			+				
21	n.t.	+	n.t.	n.t.	n.t.	+	+
22							
23							
24							
25							

a) + = few, scattered cells ++ = moderate number of cells +++ = numerous cells

n.t. = not tested; insufficient amount of tumor tissue

No cells were seen with antisera raised against ACTH, gastrin/CCK, GIP, motilin, neurotensin, secretin, or VIP

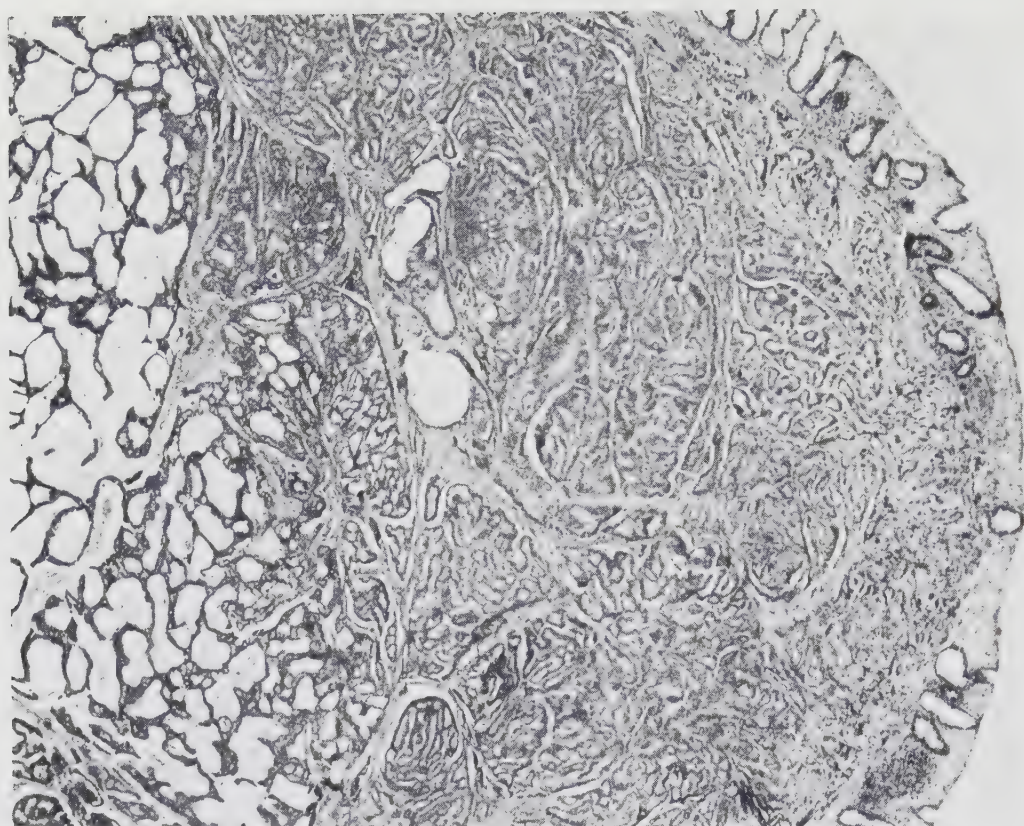


Fig. 1. Case no. 15. Rectal carcinoid situated in the mucosal and submucosal layer with ribbon-like and trabecular growth pattern. The tumor cells form anastomosing bands or loops (Soga's type E). Mucosal surface to the right. (X40). Htx-eosin staining.

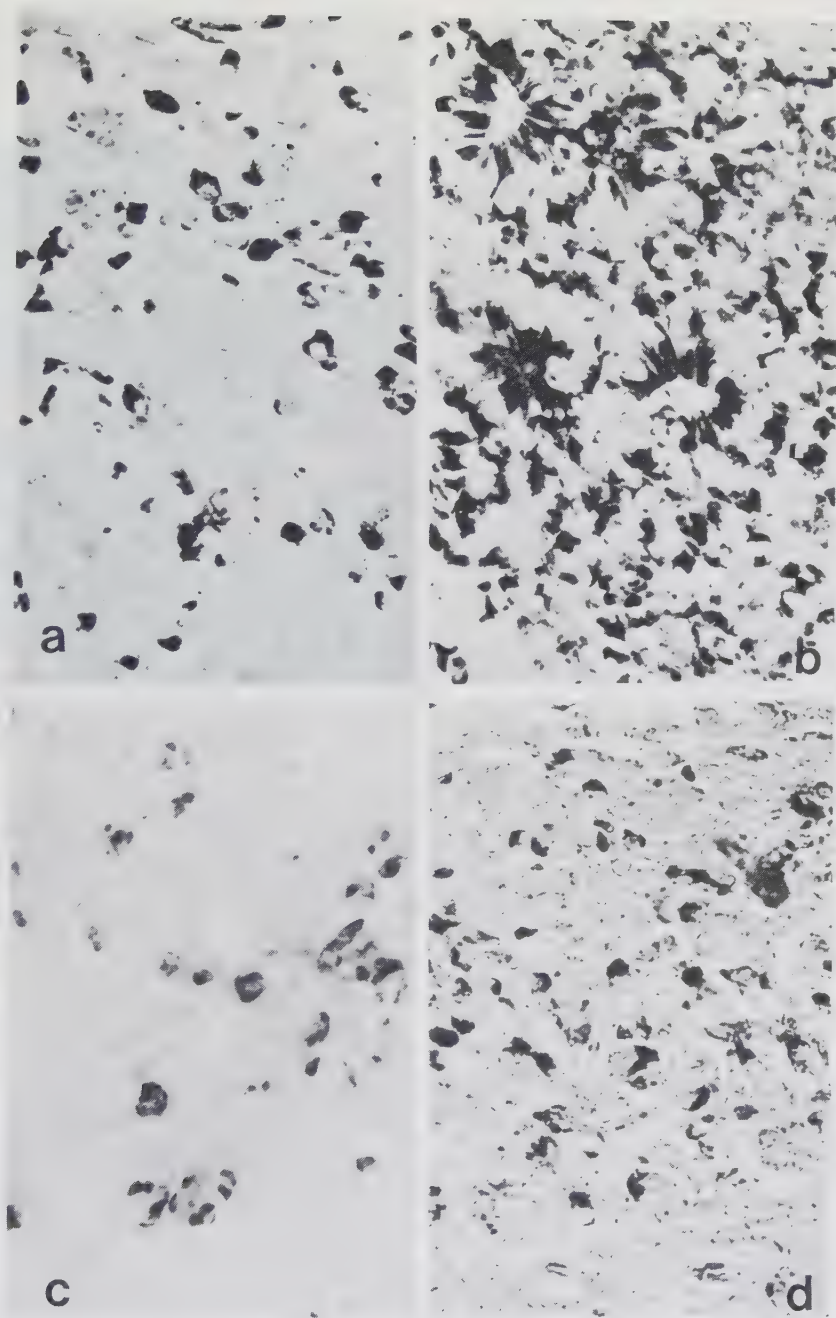


Fig. 2. a. Case no. 3. Scattered tumor cells displaying PP immunoreactivity (X250). b. Case no. 1. Area containing numerous glucagon-immunoreactive cells (X200). c. Case no. 7. Few scattered somatostatin-immunoreactive tumor cells (X200). d. Same case as in a. Fairly numerous cells display enkephalin immunoreactivity (X250). Immunoperoxidase staining.

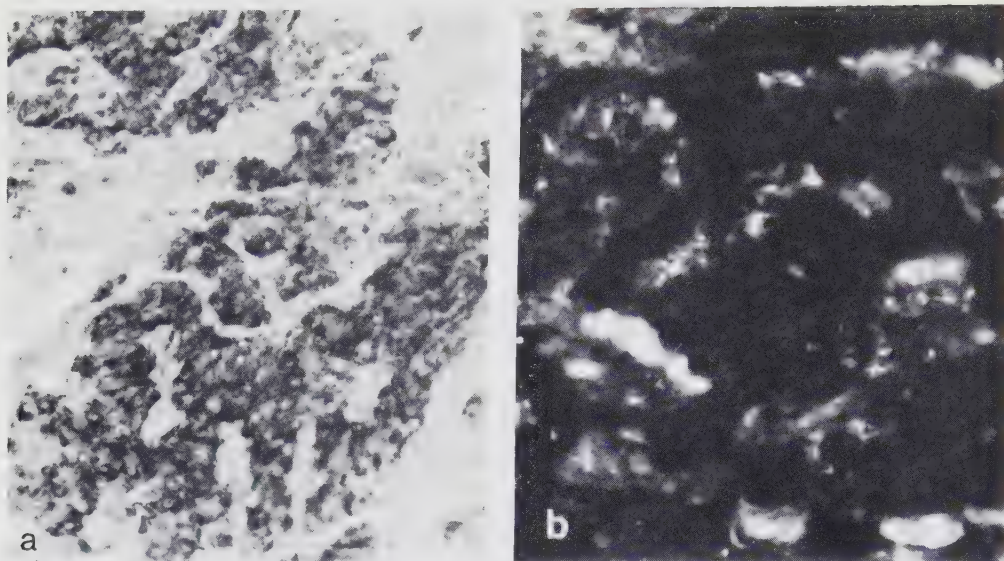


Fig. 3. a. Case no. 1 (same as in Fig. 2b). Many tumor cells display insulin immunoperoxidase staining of weak to moderate intensity (X200). b. Same case, other part of the tumor. Small clusters of cells displaying substance P immunofluorescence (X200).

NEUROHORMONAL PEPTIDES IN OVARIAN CARCINOIDS

An Immunohistochemical Study of Eighty-One Primary Carcinoids and of Intra-Ovarian Metastases from Six Mid-Gut Carcinoids

Bengt Sporrøng, MD, PhD; Sture Falkmer, MD, PhD; Stanley J. Robboy, MD, PhD; Jan Alumets, MD; Rolf Håkanson, MD, PhD; Otto Ljungberg, MD, PhD; and Frank Sundler, MD, PhD.

From the Departments of Histology, Pharmacology, Pathology and Obstetrics and Gynecology, University of Lund, Malmö General Hospital, Lund and Malmö, Sweden, and the Department of Pathology, Harvard Medical School with the James Homer Wright Pathology Laboratories, Massachusetts General Hospital, Boston, Ma., USA.

ABSTRACT

Eighty-one primary ovarian carcinoids and intra-ovarian metastases from six mid-gut carcinoids were examined for the presence of tumor cells immunoreactive with antisera raised against various neurohormonal peptides, mostly of gastro-entero-pancreatic (GEP) origin. Twenty of the primary and two of the metastatic carcinoids contained such tumor cells. The incidence of tumors with any kind of neurohormonal peptide immunoreactive tumor cells was 53% in the trabecular carcinoids, and 42% in the strumal carcinoids, whereas the incidence was much lower (7%) in the insular type. Immunoreactive pancreatic polypeptide (PP), glucagon, enkephalin, and somatostatin, were those neurohormonal peptides most commonly observed in the tumor cells of the primary carcinoids. Those less commonly found were substance P, calcitonin, VIP, neurotensin, β -endorphin, and ACTH. Four metastatic carcinoids were non-reactive with all the antisera used. Cells storing immunoreactive insulin, glucagon, PP, VIP, gastrin, substance P, or enkephalin were found in one of the two remaining metastatic carcinoids; in the other only gastrin immunoreactive tumor cells were observed. The occurrence and distribution of tumor cells storing the neurohormonal peptides in ovarian carcinoids are discussed in relation to their possible origin in the ovary and to carcinoids in the gut.

INTRODUCTION

Benign cystic teratomas of the ovary or the testis offer interesting aspects on the histogenesis of carcinoid tumors. Next to epidermoid carcinomas, the carcinoid is the most frequent neoplasm, originating in the teratoma (8). From light-microscopical analyses it has been possible to recognize three major types of primary ovarian carcinoids, *viz.* insular carcinoids (23), strumal carcinoids (26), and trabecular carcinoids (24). The growth pattern of the insular carcinoid is similar to that of the 5-HT-containing carcinoid of mid-gut origin, whereas the trabecular carcinoid resembles hind-gut carcinoids. The strumal carcinoid is composed of thyroid parenchyma together with a carcinoid component which usually is of the trabecular type (26). For comparison with primary

ovarian carcinoids, a series of pancreatic and gut carcinoids, metastatic to the ovary, had earlier been analyzed clinicopathologically (25). Their growth pattern was indistinguishable from that of primary ovarian carcinoids of insular type.

Although it is usually not possible to define from which of the teratomatous structures the primary ovarian carcinoid arises, it has, nevertheless, been generally assumed that they originate from neuroendocrine stem cells (5, 15). This assumption is supported by immunochemical and immunohistochemical demonstration of neurohormonal peptides in primary carcinoids of ovary and testis (27,28).

In a pilot study (28), comprising 73 of the present 81 primary ovarian carcinoids*¹, we observed the following incidence of tumours containing cells reactive with antisera against recognized neurohormonal peptides: Insular carcinoids 5%, trabecular carcinoids 30%, strumal carcinoids 50%. We have previously found the corresponding figure for primary carcinoids of the rectum to be 85% (3). As this discrepancy could be due to differences in the preservation, fixation, and histotechnical processing, it was essential to supplement and finalize the preliminary observations by a more comprehensive study, including additional cases, additional specimens (some of them gross specimens), carcinoids metastatic to the ovaries, and a wider spectrum of antisera.

MATERIAL AND METHODS

Cases

Eighty-one formalin-fixed primary ovarian carcinoids (42 insular, 24 strumal and 15 trabecular) and 6 formalin-fixed ovarian metastases (all insular) from mid-gut carcinoids were studied. The material was collected at surgical operations and was obtained from several hospitals. The condition of the tissue material may have been affected by operative variables such as ischemia at operation, differences in the choice of fixative, inadequate penetration of the fixative, and the age of the paraffin blocks. Some of them had been stored for 15 years. Other tissue material had been kept in formalin for several years.

*¹) "Carcinoid tumors" were identified solely by their typical growth pattern at the light-microscopic level.

The original paraffin blocks were available in 23 cases of primary ovarian carcinoids (7 insular; 9 strumal, and 7 trabecular); of these formalin-fixed gross specimens were accessible from 2 insular, 2 strumal, and 1 trabecular carcinoid. In all 6 cases of metastases, gross specimens were available in 10% formalin. In 58 cases we had access only to 3-6 slides with unstained paraffin sections. Consequently, only a limited number of antisera could be applied (cf. Table 4). Salient parts of the clinical history of each patient were obtained from the record sheets and have been presented in previous reports (8, 23, 24, 25, 26).

Antisera

All antisera used are listed in Table 1 together with their source and working dilutions. The glucagon antiserum reacts equally well with pancreatic-type and gut-type glucagon (3). The gastrin antiserum cross-reacts with CCK (17).

Immunohistochemistry

Deparaffinized sections were hydrated and washed thoroughly overnight. The sections were then processed for histochemical demonstration of the different neurohormonal peptides, listed in Table 1, either by the use of the indirect immunofluorescence method (6) or by the peroxidase-anti-peroxidase (PAP) technique (30). The antisera were applied for 3 hours (immunofluorescence) or 24 hours (PAP technique). Controls were run as recommended by Sternberger (30), and included the application of antiserum pretreated with the antigen (10-100 µg of pure or synthetic antigen per ml diluted antiserum) as specified in Table 1.

Histological Procedures

Twenty-six (20 primary; 6 metastatic) tumors, available in paraffin blocks, were examined for the presence of argyrophil and argentaffin cells by means of the Grimelius and the Masson-Hamperl techniques (8).

RESULTS

Twenty of the 81 primary ovarian carcinoids, and 2 of the 6 metas-

tatic carcinoids, contained tumor cells displaying immunoreactivity with antisera against various neurohormonal peptides (Tables 2, 3). In the primary tumors, the most common immunoreactive tumor cells were those storing PP, glucagon, enkephalin, or somatostatin-like material. Next in frequency were tumors containing substance P, VIP, or calcitonin. ACTH and β -endorphin immunoreactive cells were found in one tumor each. The metastatic carcinoids were too few to permit an evaluation of the frequency of occurrence of the various neurohormonal peptides. Two of the tumors harbored immunoreactive cells that stained with the gastrin/CCK antiserum (not observed in any of the primary carcinoids); one of them contained 5 additional types of immunoreactive tumor cells, including even insulin. Neither in the primary, nor in the metastatic carcinoids were secretin or motilin immunoreactive cells found.

The incidence of tumors with any kind of neurohormonal immunoreactive tumor cells was 53% in the trabecular carcinoids, and 42% in the strumal carcinoids. The incidence was much lower (7%) in the insular type.

Immunoreactive tumor cells invariably constituted a minor fraction of the total tumor mass. The different peptide-containing tumor cells found varied greatly in number and distribution in the individual carcinoids. The tumor cells reacting with antisera against glucagon, PP, enkephalin, or calcitonin often had a patchy distribution when they occurred in abundance (Fig. 1). When few, they were more uniformly distributed. Tumor cells, storing somatostatin, VIP, gastrin/CCK, substance P, neurotensin, or β -endorphin were diffusely scattered in the tumor parenchyma, also when they were numerous (Fig. 2).

Only 6 of all the carcinoids investigated were associated with the carcinoid syndrome. Three were primary (insular type) and 3 were metastatic. Of these, one primary tumor contained cells reacting with antisera against PP and enkephalin, and one metastatic carcinoid harbored tumor cells reacting with antisera against insulin, glucagon, PP, VIP, gastrin/CCK, substance P, and enkephalin. The remaining 4 carcinoids did not stain with the antisera tested.

In 23 primary ovarian carcinoids the correlation between argyrophilia and immunoreactivity was studied. Argyrophil tumor cells were found

in 18 carcinoids and immunoreactive cells in 10; 9 of these 10 contained argyrophil as well as immunoreactive cells. The remaining non-argyrophil carcinoid was of trabecular type with somatostatin and glucagon immunoreactive tumor cells.

Analogous studies of the correlation between argentaffinity and immunoreactivity were invalidated by the fact that only 4 of the primary ovarian carcinoids were argentaffin. Two were of insular type, one was trabecular, and one strumal. One of the insular carcinoids and the trabecular one were both devoid of immunoreactive tumor cells. The other insular carcinoid was immunoreactive with antisera against glucagon, PP, neurotensin, enkephalin, and ACTH, whereas the argentaffin strumal carcinoid contained somatostatin immunoreactive tumor cells only.

The results of these correlative studies thus show that argyrophilia and argentaffinity are poor indicators of the presence or absence of GEP neurohormone immunoreactive tumor cells in primary ovarian carcinoids.

DISCUSSION

Peptide hormone producing tumors occur in a variety of epithelial organs such as the anterior pituitary, the bronchial tree, the thyroid, digestive tract and pancreas, as well as in the thymus and genitourinary tract (18). Most of our knowledge concerning the production of neurohormonal peptides in carcinoids originates from immunohistochemical and immunochemical investigations on endocrine tumors of the digestive tract or bronchial tree. Many of these tumors are heterogenous in that they contain several different cell types, each producing its own peptide (6), or cell types that store several immunoreactive peptides (16, 29).

Earlier immunohistochemical investigations on primary ovarian carcinoids have demonstrated calcitonin (7, 12) as well as triiodothyronine and thyroxine (14) in strumal-type carcinoids. Calcitonin has also been found in a trabecular carcinoid (24), and recently substance P occurred in lymph node metastases from a primary ovarian carcinoid of insular type (27). So far, there has been no systematic search for neurohormonal peptides in primary ovarian carcinoids.

The present material comprises tumors fixed under non-optimal conditions. This must be considered when the results are evaluated. A broader spectrum of peptides and a higher frequency of immunoreactive cells would certainly have been obtained with improved sampling conditions and histochemical processing. Tumor cells, immunoreactive with substance P antisera, for instance, should theoretically be expected to appear at a much higher frequency (27, 32), but this peptide appears to be particularly vulnerable to a poor histochemical processing (Sundler, unpublished observations). The situation may be the same for other peptides and for amines, such as 5-HT, thus explaining the low incidence of argentaffin cells. In addition, only a limited number of antisera could be applied to those tumors from which only paraffin sections were available. They, too, might have contributed to a higher frequency of tumors with immunoreactive cells if enough material had been accessible for testing all the antisera available.

Already the light microscopic appearance of the tumors (growth pattern, cell morphology, staining characteristics) suggests a close relationship between carcinoid tumors in the gut and carcinoid tumors of the ovary.

All the neurohormonal peptides detected in primary ovarian carcinoids have previously been demonstrated in gastro-intestinal and/or pancreatic endocrine tumors, particularly in those originating in the rectum and sigmoid colon (1, 2, 3, 9, 16). Moreover, ovarian carcinoids resemble gut endocrine tumors in that several different immunoreactive peptides may occur in the same tumor (16). A similar spectrum of peptides was observed in an analogous study of 53 carcinoids arising in benign testicular teratomas, where 11 (21%) contained tumor cells positive to antisera against PP, somatostatin, or glucagon (5).

Gut endocrine tumors are thought to arise from peptide hormone-producing cells, which are normal constituents of the intestinal epithelium. However, in the female genital tract, such cells occur only in the vaginal, and, on rare occasions, in the endocervical epithelium (19). Primary ovarian carcinoids probably arise from peptide hormone producing cells which occur in teratomatous tissue. Alternatively, cells programmed to produce neurohormonal peptides arise in the

neural crest and migrate to the ovary where they later give rise to a carcinoid tumor (20, 21).

The difference between the production of neurohormonal peptides in primary ovarian carcinoids and in the metastases to the ovary shows that the metastases probably retain the same neurohormonal peptide production as the original tumor in the mid-gut (see below). However, our small material does not allow any definite conclusions in this respect.

Within the primary ovarian carcinoids, the incidence of neurohormonal producing cells in the trabecular and the strumal types is similar, whereas the incidence in the primary insular type is considerably lower. This may indicate that the trabecular and the strumal types are identical with respect to the production of neurohormonal peptides, and that they differ from the insular type by being closely related to the rectal carcinoids, not only in their growth pattern but also immunohistochemically. The low incidence of immunoreactive tumor cells in the insular ovarian carcinoid may represent a genuine difference supporting the idea that the latter tumors are more closely related to mid-gut carcinoids, where often surprisingly few immunoreactive tumor cells usually are observed (2). As, theoretically, the incidence of peptide hormone producing cells should be 100% in all carcinoid tumors, the small number of immunoreactive tumor cells detected indicates that the main peptides produced in mid-gut carcinoids have not yet been isolated and characterized.

A preliminary report (28) has been given at the International Symposium "Cellular Basis of Chemical Messengers in the Digestive System", held in Santa Monica, Calif., Jan 16-18, 1980.

ACKNOWLEDGEMENTS

Supported by grants from the Swedish Medical Research Council (Projects No. 04X-4499 and 12X718), the Swedish Cancer Society (Project No. 806-5XA), the Cancer Research Foundation at Malmö General Hospital, and the Faculty of Medicine at the University of Lund.

TABLE 1. Specification of the GEP neurohormonal peptide antisera used and their source of origin and working dilutions.

Antisera raised against	Working IF *)	Dilution PAP **)	Code	Source
Pure bovine insulin	1/80	1/5120	LAI	L.G. Heding, Novo Res.Inst., Bagsvaerd, Denmark
Synthetic ovine somatostatin (SOM)		1/2560	19578	M.P. Dubois, Station Physiol.Reprod., INRA, Nouzilly, France
Pure porcine glucagon		1/640	7811	J.E. Thorell, Dept.Nucl.Med., Malmö Gen. Hospital, Malmö, Sweden
Pure bovine pancreatic polypeptide (PP)	1/320	1/2560	7823	J.E. Thorell, Dept.Nucl.Med., Malmö Gen. Hospital, Malmö, Sweden
Pure porcine secretin	1/80	1/2560	5585	O.B. Schaffalitzky de Muckadell, Bispebjerg Hospital, Copenhagen, Denmark
Pure porcine vasoactive intestinal polypeptide (VIP)		1/5120	5603	J.Fahrenkrug, Dept.Clin.Chem., Copenhagen County Hospital, Glostrup, Denmark
		1/5120	7852	J.E. Thorell, Dept.Nucl.Med., Malmö Gen.Hospital, Malmö, Sweden
Synthetic human gastrin 2-17		1/5120	4562	J.F. Rehfeld, Dept.Med.Biochem., University Aarhus, Aarhus, Denmark
	1/20		K 16	G.Nilsson, Dept.Pharmacol., Karolinska Inst., Stockholm, Sweden
Synthetic bovine substance P (SP)				
	1/80		SP-8	P.C. Emson, Med.Res.Council, Cambridge, England
Synthetic bovine neurotensin (NT)		1/640	HC-8	R.E. Carraway, Lab.Hum.Reprod., Dept.Physiol., Harvard Med.Sch., Boston, Ma, USA
Synthetic leu- or met-enkephalin (Enk)		1/240		
		1/640	Leu-enk Met-enk	R.J.Miller & K.J. Chang, Burroughs Wellcome Res. Lab., Triangle Park, NC, USA
Synthetic human β -endorphin (β -End)		1/640	7763	J.E. Thorell, Dept.Nucl.Med., Malmö Gen.Hospital, Malmö, Sweden
(β -lipotropin 61-91)				
Synthetic porcine motilin		1/640	MBR-1105	N. Yanaihara, Shizuoka Coll.Pharm., Shizuoka, Japan
Synthetic human calcitonin (CT)		1/160	AS292/5	I. MacIntyre, Endocr.Unit, Royal Postgrad.Med. Sch., London, England
Pure porcine adrenocorticotrophic hormone (ACTH)	1/80	1/640	No. 1	Own

*) IF: Immunofluorescence **) PAP: Peroxidase-Anti-Peroxidase procedure

COMMENTS TO TABLE 1:

The antisera were inactivated by the respective antigen. The antigens were obtained from the following sources:

Insulin and glucagon were both kindly donated by Dr L.G. Heding, Novo Research Institute, Bagsvaerd, Denmark.

Somatostatin, substance P, enkephalin, β -endorphin, and calcitonin, were all purchased from Peninsula Inc., San Carlos, Calif., USA.

BPP was generously supplied by Dr R.E. Chance, Lilly Res. Lab., Indianapolis, Indiana, USA.

Secretin, VIP, and motilin, were kindly provided by Prof. V. Mutt, Dept. of Biochem. II, Karol. Inst., Stockholm, Sweden.

Gastrin-17 was donated by Prof. J.F. Rehfeld, Dept. of Med. Biochem., University of Aarhus, Aarhus, Denmark.

Neurotensin was bought from U.C.B., Brussels, Belgium.

ACTH was purchased from Ferring Inc., Malmö, Sweden.

TABLE 2: Number of Tumors, containing Cells Reactive with Antisera against Neurohormonal Peptides in the Three Different Groups of Primary Ovarian Carcinoids and in the Intra-Ovarian Carcinoid Metastases of Mid-Gut Origin.

As further shown in Table 3, most of the carcinoids with any kind of neurohormonal immunoreactive tumor cells were of multihormonal type. The majority of the carcinoids in each of the four groups did not contain any neurohormonal immunoreactive tumor cells.

Same abbreviations of the neurohormonal peptides as in Table 1.

The figures within brackets give the number of carcinoids tested, *versus* those displaying immunoreactivity, in those 58 of the primary ovarian carcinoids where only a limited number of sections were available. As to the 6 metastatic tumors, all were tested with all antisera. The same applies to those 23 primary carcinoids where gross specimens and/or paraffin blocks were available.

Type of carcinoid	Total number of tumors	Number of tumors harboring immuno-reactive cells	Number of tumors containing cells reactive with antisera against											
			Insulin	SOM	Glucagon	PP	VIP	Gastrin/CCK	SP	NT	Enk	B-End	CT	ACTH
Insular	42(35)	3 (7%)		1(30/1)	1(29/0)	2(22/0)	(2/0)		1(19/0)	2(32/0)	(0)			
Trabecular	15(8)	8 (53%)		4(7/1)	4(5/0)	3(6/2)	1	(0)	2	1(3/0)	2(6/1)	2(0)	1	
Strumal	24(15)	10(42%)		1(7/0)	4(0)	9(8/4)	1	(1/0)	1	(2/0)	3(5/1)	1	2(12/2)	
Metastatic	6	2	1		1	1		2	1		1			
Total	87	23(26%)	1	6	10	15	2	2	4	2	8	1	4	1

TABLE 3: Number of Immunoreactive Neurohormonal Peptides Found in Tumor Cells of Each of the Primary and Metastatic Ovarian Carcinoids (cf. Table 2).

Type of carcinoid	Total number of carcinoids	Number of immunoreactive neurohormonal peptides found						
		None	1	2	3	4	5	6
Insular	42	39	1	1		1		
Trabecular	15	7	2	3	2		1	
Strumal	24	13	3	4	4			
Metastatic	6	4	1					1
Total	87	63	7	8	6	1	1	1

TABLE 4: Summary of Immunohistochemical Findings in the 58 Primary Ovarian Carcinoids from which only a Limited Number of Sections were available.

Same abbreviations of the neurohormonal peptides as in Tables 1 and 2.

Type of carcinoid	Number of tumors	Number of carcinoids tested/ <i>vs.</i> those containing tumor cells displaying immunoreactivity with antisera raised against:						
		SOM	Glucagon	PP	Gastrin/CCK	NT	Enk	CT
Insular	35	30/1	29/0	22/0	2/0	19/0	32/0	0
Trabecular	8	7/1	5/0	6/2	0	3/0	6/1	0
Strumal	15	7/0	0	8/4	1/0	2/0	5/1	12/2
Total	58	44/2	34/0	36/6	3/0	24/0	43/2	12/2

REFERENCES

1. Alumets, J., Falkmer, S., Grimelius, L., Håkanson, R., Ljungberg, O., Sundler, F. and Wilander, E.: Immunocytochemical demonstration of enkephalin and β -endorphin in endocrine tumors of the rectum. A survey of 27 colo-rectal carcinoids. *Acta Path Microbiol. Scand. Sect. A*, 88:103-109, 1980.
2. Alumets, J., Falkmer, S., Håkanson, R., Ljungberg, O. and Sundler, F.: Characteristic spectrum of neurohormonal peptides in endocrine tumours arising in foregut, midgut or hindgut derivatives. *Regul. Pept. 1*, Suppl. 1:S3, 1980.
3. Alumets, J., Alm, P., Falkmer, S., Håkanson, R., Ljungberg, O., Mårtensson, H., Sundler, F. and Tibblin, S.: Immunohistochemical evidence of peptide hormones in endocrine tumors of the rectum. *Cancer* (In press) 1981.
4. Bosman, F.T. and Louwerens, J.-W.: APUD cells in teratomas. *J. Histochem. Cytochem.* 28:617-618, 1980.
5. Brodner, O.G., Grube, D., Helmstaedter, V., Kreienbrink, U.L., Wurster, K. and Forssmann, W.G.: Endocrine GLP-cells in primary testicular teratoma. *Virch. Arch. A. Path. Anat. and Histol.* 388: 251-262, 1980.
6. Coons, A.H., Leduc, E.H. and Conolly, J.M.: Studies on antibody production. I: A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* 102:49-60, 1955.
7. Dayal, Y., Tashjian, A.H. and Wolfe, H.J.: Immunocytochemical localization of calcitonin-producing cells in a strumal carcinoid with amyloid stroma. *Cancer* 43:1331-1338, 1979.
8. Falkmer, S., Frankendal, B., Hassler, O. and Ångström, T.: Carcinoid tumour in a benign cystic teratoma of the ovary. *Acta Path. Microbiol. Scand. Sect. A*, 80:Suppl. 233:91-97, 1972.
9. Falkmer, S., Alumets, J., Håkanson, R., Ljungberg, O., Sundler, F. and Tibblin, S.: Occurrence of pancreatic polypeptide (PP), somatostatin, glucagon, insulin, enkephalin, β -endorphin, and substance P cells in rectal carcinoids. A preliminary report of nineteen cases. In: Gut Peptides. Secretion, Function and Clinical Aspects. A. Miyoshi, Eds., Amsterdam, Elsevier/North-Holland, 1979; pp. 351-355.
10. Fox, H., Kazzaz, M. and Langely, F.A.: Argyrophil and argentaffin cells in the female genital tract and in ovarian mucinous cysts. *J. Path. Bact.* 88:479-488, 1964.

11. Gloor, E.: T ratomes matures b nins avec tumeur maligne et t ratomes monodermiques malins de l'ovaire. Pr sentation anatomo-clinique de 10 cas. *Schweiz. Med.Wschr.* 109:968-975, 1979.
12. Greco, M.A., LiVolsi, V.A., Pertschuk, L.P. and Bigelow, B.: Strumal carcinoid of the ovary. An analysis of its components. *Cancer* 43:1380-1388, 1979.
13. H kanson, R., Alumets, J., Ekman, R., Sundler, F. and Thorell, J.I.: Peptides common to carcinoid tumour granules and enterochromaffin cells. *Regul. Pept.* 1, Suppl. 1:S46, 1980.
14. Hasleton, P.S., Kelehan, P., Whittaker, J.S., Burslem, R.W. and Turner, L.: Benign and malignant struma ovarii. *Arch.Path.Lab. Med.* 102:180-184, 1978.
15. Kunze, E., Schauer, A., Droese, M., Geiger, R. and Urbanczyk, H.: Das prim re endokrin aktive Ovarialkarzinoid. *Verhandl.Dtsch.Ges. Path.* 61:139-143, 1977.
16. Larsson, L.-I., Grimelius, L., H kanson, R., Rehfeld, J.F., Stadil, F., Holst, J.J., Angervall, L. and Sundler, F.: Mixed endocrine pancreatic tumors producing several peptide hormones. *Am.J.Path.* 79:271-284, 1975.
17. Larsson, L.-I., and Rehfeld, J.F.: Localization and molecular heterogeneity of cholecystokinin in the central and peripheral nervous system. *Brain Res.* 165:201-218, 1978.
18. Marks, C.: Carcinoid tumors: A clinicopathologic study. Boston, Mass.: G.K: Hall, 1979; pp. 1-154.
19. Mullins, J.D. and Hilliard, G.D.: Cervical carcinoid ("argyrophil cell"carcinoma) associated with an endocervical adenocarcinoma: A light and ultrastructural study. *Cancer* 47:785-790, 1981.
20. Pearse, A.G.E. and Polak, J.M.: Endocrine tumours of neural crest origin: Neuroblastomas, APUDomas and the APUD concept. *Med.Biol.* 52:3-18, 1974.
21. Pearse, A.G.E.: The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic, and pathologic implications of the concept. *J. Histochem. Cytochem.* 17:303-313, 1969.
22. Riley, P.A. and Sutton, P.M.: Why are ovarian teratomas benign, whilst teratomas of-the testis are malignant? *Lancet* I:1360,1975.
23. Robboy, S.J., Norris, H.J. and Scully, R.E.: Insular carcinoid primary in the ovary. A clinicopathologic analysis of 48 cases. *Cancer* 36:404-418, 1975.

24. Robboy, S.J., Scully, R.E. and Norris, N.J.: Primary trabecular carcinoid of the ovary. *Obstetr.Gyn.* 49:202-207, 1977.
25. Robboy, S.J., Scully, R.E. and Norris, N.J.: Carcinoid metastatic to the ovary. A clinicopathologic analysis of 35 cases. *Cancer* 33:798-811, 1974.
26. Robboy, S.J. and Scully, R.E.: Strumal carcinoid of the ovary: An analysis of 50 cases of a distinctive tumor composed of thyroid tissue and carcinoid. *Cancer* 46:2019-2034, 1980.
27. Skrabanek, P., Dervan, P., Cannon, D. and Powell, D.: Substance P in ovarian carcinoid. *J.Clin. Path.* 33:160-162, 1980.
28. Sporrang, B., Falkmer, S., Robboy, S.J., Alumets, J., Håkanson, R., Ljungberg, O. and Sundler, F.: Evidence for neurohormonal peptides in ovarian carcinoids. A preliminary report of immunohistochemical findings. In: *Cellular Basis of Chemical Messengers in the Digestive System*, M.I. Grossman, M.A.B. Brazier, and J. Lechago, Eds., New York, Academic Press, *UCLA Forum in Med.Sci.* 23:257-265, 1981.
29. Sundler, F., Alumets, J., and Håkanson, R.: Majority and minority cell populations in GEP and bronchial endocrine tumors. *Scand.J.Gastroent.* 14:Suppl. 53:9-13, 1979.
30. Sternberger, L.A.: *Immunocytochemistry*. (Ed.2) New York: John Wiley & Sons, pp. 1-354, 1979.
31. Thorson, Å., Hanson, A., Pernow, M., Söderström, N., Waldenström, J., Winblad, S. and Wulff, H.B.: Carcinoid tumour within an ovarian teratoma in a patient with the carcinoid syndrome (carcinoidosis). Clinical picture and metabolic studies before and after total resection of tumour. *Acta Med.Scand.* 161:495-505, 1958.
32. Wilander, E., Portela-Gomes, G., Grimelius, L., Lundqvist, G. and Skoog, V.: Enteroglucagon and substance-P-like immunoreactivity in argentaffin and argyrophil rectal carcinoids. *Virch. Arch.B.:Cell Path.* 25:117-124, 1977.

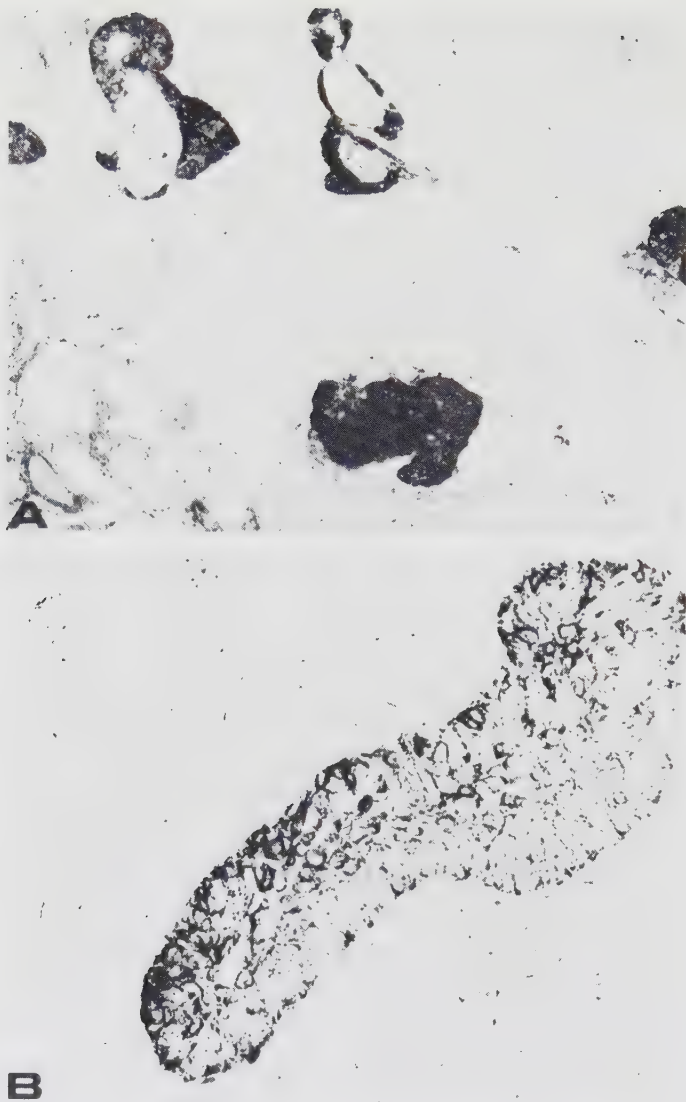


FIG. 1. Medium-power photomicrographs of PAP-stained sections of two primary ovarian carcinoids of strumal type, showing the patchy distribution of enkephalin (FIG. 1A) and calcitonin (FIG. 1B) immunoreactive tumor cells in the neoplastic parenchyma.

This distribution pattern was typical for those carcinoid tumor cells that reacted with antisera against glucagon, PP, enkephalin, and calcitonin, when they occurred in abundance. When few, they were more uniformly distributed (as shown in FIG. 2).

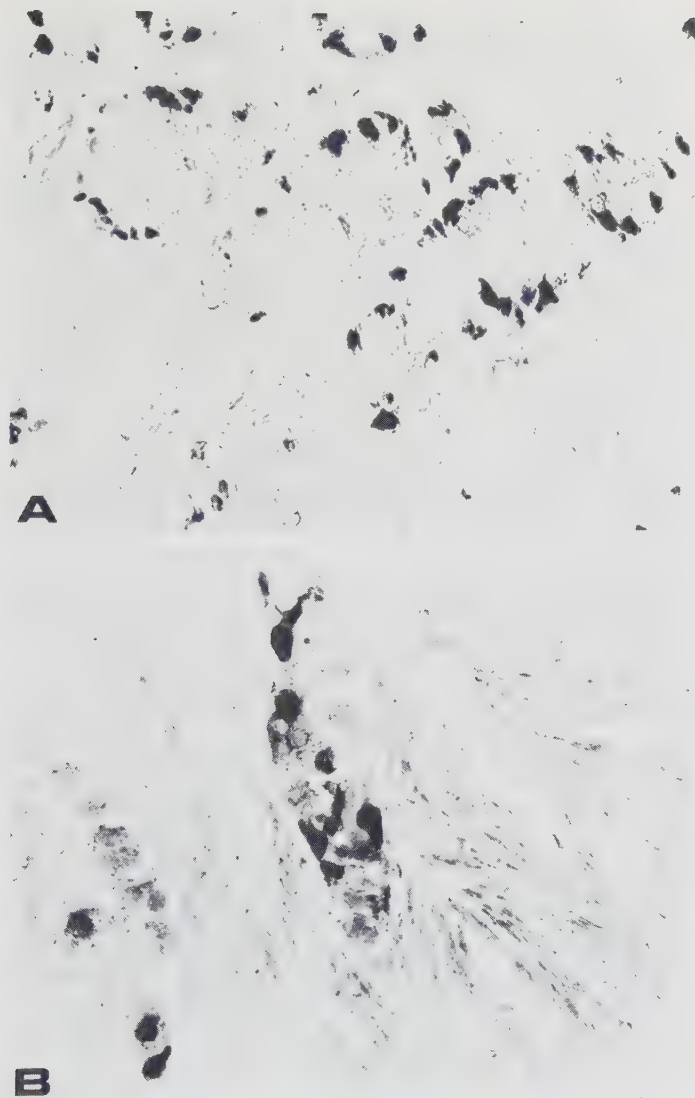


FIG. 2. Medium-power photomicrographs of PAP-stained sections of two primary ovarian carcinoids of strumal type, showing the more uniform distribution pattern observed in tumor cells immunoreactive with antisera against the pancreatic polypeptide (PP) (FIG. 2A) and somatostatin (FIG. 2B).

This distribution pattern was typical for carcinoid tumor cells storing not only PP (when few; cf. FIG. 1) and somatostatin, but also VIP, gastrin/CCK, substance P, neurotensin, and β -endorphin (also when they were abundant).

NEUROHORMONAL PEPTIDE IMMUNOREACTIVE CELLS IN MUCINOUS
CYSTADENOMAS AND CYSTADENOCARCINOMAS OF THE OVARY

Bengt Sporrang, Jan Alumets, Li Clase, Sture Falkmer,
Rolf Håkanson, Otto Ljungberg and Frank Sundler

Departments of Histology, Pharmacology, Pathology, and
Obstetrics and Gynecology, University of Lund, Malmö
General Hospital, Lund and Malmö, Sweden

SUMMARY

In 144 benign mucinous cystadenomas of the ovary, 33 mucinous cystadenomas of borderline malignancy, and 64 mucinous cystadenocarcinomas, the incidence of tumours containing argyrophil (probably endocrine) cells was 18%, 33%, and 53%, respectively. The results of a semiquantitative assessment of the number of argyrophil cells in each individual tumour indicates that the greatest numbers occurred in the cystadenocarcinomas. As, however, the tumour cell density was larger in the cystadenocarcinomas than in the cystadenomas, and as the argyrophil cells often had a patchy distribution in the tumour epithelium, the incidence figures are unreliable. In addition, the visualization of the argyrophil cells depends on an adequate fixation which is difficult to achieve in the routine processing of large tumour specimens.

Many argyrophil cells in the cystadenocarcinomas displayed immunoreactivity with antisera raised against gastro-entero-pancreatic (GEP) neurohormonal peptides. In ten such tumours immunohistochemical evidence was obtained for the presence of the following neurohormonal peptides in the tumour cells: somatostatin, glucagon, gastrin/CCK, neurotensin, and enkephalin. Four of these ten cystadenocarcinomas were multihormonal, in that three contained two cell populations storing GEP neurohormonal peptides, and one tumour even three such populations. In the benign cystadenomas, however, no immunoreactive tumour cells were found. In those of borderline type, only two harboured immunoreactive cells. In both cases the tumour cells stored gastrin/CCK.

The general appearance of the epithelium in the mucinous tumours - a continuous single-cell layer of mucin-producing cells intermingled with argyrophil cells of open type - and the spectrum of neurohormonal peptides observed, indicate an origin from the foregut endoderm.

INTRODUCTION

Both argyrophil and argentaffin cells have been demonstrated in the epithelium of mucinous cystadenomas and cystadenocarcinomas of the human ovary (Fox et al., 1964; Klemi, 1978). Ultrastructurally, these cells are characterized by the presence of small secretory granules (Schmid, 1977; Klemi and Nevalainen, 1978). In the gastro-entero-pancreatic (GEP) region such cell types are known to produce neurohormonal peptides (cf. Creutzfeldt, 1980). Tumours arising from these cells (insulomas; carcinoids) (Solcia, 1980) may evoke symptoms of endocrine disorder, for instance the insulinoma syndrome, the Zollinger-Ellison syndrome, and the carcinoid syndrome (Friesen and Bolinger, 1978; Grahame-Smith, 1979). Despite the fact that primary ovarian mucinous tumours contain cells with a peptide hormone producing potential, they are usually not associated with endocrine symptoms (Scully, 1980). In two histopathologically documented cases, however, a Zollinger-Ellison syndrome was found to be associated with a mucinous cystadenocarcinoma (Cocco and Conway, 1975) and a mucinous cystadenoma (Long et al., 1980); both tumours were found to produce gastrin. Carcinoid tumours may also arise in the ovary. Recently, it was shown that such tumours contain cells, reacting with antisera against a variety of neurohormonal peptides, though usually not associated with endocrine symptoms (Sporrong et al., 1981 a,b; Dayal et al., 1980).

These observations prompted a more systematic search for neurohormonal peptides in ovarian mucinous tumours.

MATERIAL AND METHODS

A re-examination was made of all the ovarian mucinous cystadenomas and cystadenocarcinomas available from the 1970-79 files of the Department of Pathology at the Malmö General Hospital, using the Grimelius (1968) silver nitrate procedure for detecting argyrophil tumour cells. Of the 241 tumours examined (paraffin sections from formalin-fixed specimens), 71 contained argyrophil cells. These 241 tumours were grouped according to the World Health Organization classification of ovarian tumours (Serov and Scully, 1973) into three categories: benign cystadenomas (II a); cystadenomas of

borderline type (II b); and cystadenocarcinomas (II c); the corresponding number of tumours was 144, 33 and 64, respectively.

For immunohistochemistry, sections of 5 μ m thickness, were deparaffinized, hydrated and washed over night. The antisera against neurohormonal peptides were applied for 3 h at room temperature when the immunofluorescence procedure was used. The antisera used and their source and dilutions are listed in Table 1. The antiserum against gastrin reacts equally well with cholecystokinin (CCK), and that against glucagon reacts with both gut type and pancreatic type glucagon. Fluoresceinated and unlabelled sheep anti-rabbit-IgG (or anti-guinea-pig IgG) was applied as the second antibody, diluted 1:20. For immunofluorescence, the sections were rinsed and mounted in buffered glycerine. Alternatively, the peroxidase-antiperoxidase (PAP) procedure (Sternberger, 1979) was applied, using the same antisera at the dilutions given in Table 1. Incubation time was 24 hrs at +4°C. Control sections were exposed to antiserum inactivated with excess amount of antigen (10-100 μ g of synthetic and/or highly purified natural peptide per ml diluted antiserum). Details concerning antisera and antigens have previously been reported (Sporrong et al., 1981 b).

In some instances, sections first examined for the presence of immunofluorescent cells were restained with silver nitrate according to the Grimelius procedure.

RESULTS

Incidence of Argyrophil Tumour Cells. The three categories of tumours differed with regard to their apparent incidence of argyrophil epithelial cells (Table 2). Of the 144 tumours classified as benign cystadenomas, 26 (18%) harboured argyrophil cells. Eleven of the 33 tumours classified as borderline tumours contained argyrophil cells (33%), whereas 34 of the 64 cystadenocarcinomas (53%) contained such cells. When, however, these incidence figures were related to the cell density, it was found that the number of cells in the sections varied considerably; the tumour cells were 10-100 times more numerous in the borderline and malignant tumours than in the benign cystadenomas. The different incidence of argyrophil cells may perhaps partly reflect differences in the tumour cell

density. Another source of error in the assessment of the incidence of argyrophil cells was their very patchy distribution. Lastly, all the tumours were large; hence, it might be suspected that the fixation of the deeper parts of the tumour parenchyma was far from optimal. Both the argyrophil reaction and the immunoreaction depend on an adequate fixation. Thus, the incidence figures do not allow any far-reaching conclusions.

In general, the epithelium of the ovarian mucinous tumours formed a continuous single layer of mucin-producing cells, reminiscent of the epithelium seen, for instance, in the gut, in the antrum of the stomach, in the gall bladder, in the bile ducts or in the large pancreatic ducts. This light-microscopical impression was supported by the presence of disseminated argyrophil cells, usually of open type, in the epithelial lining. Some examples of argyrophil cells and a schematic outline of their rather widely varying structure are given in Figs. 1 and 2.

The number of argyrophil cells observed in the individual tumours also seemed to vary in proportion to the degree of malignancy. Thus, in the tumours classified as mucinous cystadenomas and borderline tumours argyrophil cells were few or moderate in number, whereas in the cystadenocarcinomas they were often quite numerous.

Incidence of Peptide Immunoreactive Tumour Cells. Of the tumours classified as benign cystadenomas, none were seen to harbour immunoreactive epithelial cells. Of the borderline tumours, 2 (18%) contained cells reacting with the antiserum against gastrin/CCK (Table 3). Characteristically, the immunoreactive cells were scattered in the epithelium, often two or three together (Fig. 3). Using the restaining technique, it could be shown that these gastrin/CCK-immunoreactive tumour cells were argyrophil. In both these tumours the immunoreactive cells were much fewer than the argyrophil ones.

In the cystadenocarcinoma group, 10 (29%) tumours contained neurohormonal peptide immunoreactive cells (Table 3). Six of these tumours harboured cells reacting with antiserum against only one of the neurohormonal peptides (somatostatin, glucagon/enteroglucagon, gastrin/CCK or enkephalin), whereas 3 tumours contained two

immunoreactive cell populations and one tumour even three (Table 3). The immunoreactive peptides most often found were gastrin/CCK and somatostatin, followed by enkephalin. Glucagon immunoreactive cells were found in one tumour only, whereas neurotensin immunoreactive cells occurred in another tumour which also harboured gastrin/CCK cells and enkephalin cells. Of the other cystadenocarcinomas, storing more than one demonstrable peptide, two contained cells reacting with antisera against somatostatin or gastrin/CCK, whereas one tumour harboured cells reacting with antisera against gastrin/CCK or enkephalin (Table 3). While the somatostatin immunoreactive cells were non-argyrophil, the glucagon and gastrin/CCK immunoreactive cells were argyrophil. As a rule, immunoreactive cells were less numerous than argyrophil cells.

Clinical Aspects. The histopathological analysis of the tumour material was followed by a review of the case histories of 11 of the 12 patients with tumours containing peptide immunoreactive cells. The case history of one patient (case 4) was not available. All patients, except one (case 1 ; Table 3), presented with rather non-characteristic symptoms, usually abdominal distension, of 3-6 months' duration. The patient "case 1" presented with symptoms of severe gastritis of 3 months' duration. X-ray examination failed to reveal gastric or duodenal ulcer but showed a pelvic tumour. All gastric symptoms disappeared after excision of a large cystadenoma of borderline malignancy, rich in gastrin immunoreactive tumour cells (Table 3).

DISCUSSION

A characteristic feature of many cell types producing neurohormonal peptides is their argyrophilia by the Grimelius procedure. Consequently, the presence of argyrophil cells in ovarian mucinous cystadenomas and cystadenocarcinomas suggests the ability of these types of tumours to produce such peptides. The results of the present study confirm that they can produce numerous neurohormonal peptides. As a rule, the existence of argyrophil cells in the tumour did not seem to be correlated with the age of the patients or to have any bearing on their symptoms or survival rate, as found by Fox et al. (1964) and Klemi (1978).

There are several possible explanations why such tumours usually do not give rise to clinical symptoms. The number of peptide producing cells may be so low that the serum concentration of the peptide remains within the normal range, or the rise is too small or too brief to produce overt symptoms; the peptide, although actively secreted, might be metabolized so efficiently that normal serum concentrations are maintained; or the released peptide might be immunoreactive but functionally inactive. Another intriguing possibility that might explain the paucity of symptoms, is the not infrequent occurrence of tumour cells with somatostatin-like immunoreactivity together with other neurohormonal immunoreactive substances, notably gastrin/CCK-like activity. Somatostatin is a well known inhibitor of, for instance, the release of gastrin (Lefendić, 1980; McCann et al., 1980). It is by no means unlikely that it would be possible to find an elevated concentration of a neurohormonal peptide in serum if a blood sample could be drawn from the ovarian vein in a patient with a mucinous cystadenocarcinoma of the ovary. Some support for this suggestion comes from the case history of patient "case 1" (Table 3), where there seemed to be a correlation between the sudden disappearance of severe gastritis symptoms and the removal of a gastrin/CCK-producing tumour.

It is also worth noting that the spectrum of the immunoreactive peptides observed in these mucinous cystadenomas differs from that observed by immunohistochemistry in ovarian carcinoids (Sporrøng et al., 1981 a,b). In the latter group of tumours, pancreatic polypeptide (PP) was found to be the most common neurohormonal peptide, followed by glucagon/enteroglucagon and enkephalin. In an analogous study of rectal carcinoids (Alumets et al., 1981), tumour cells, displaying immunoreactivity with antisera against PP, glucagon, somatostatin, insulin, substance P, enkephalin, or β -endorphin, were observed (in decreasing order of frequency). The reason for these discrepancies is unknown, but they might be due to differences in technique. Rectal carcinoids are usually small and easily accessible for immediate microscopical investigations (Alumets et al., 1981), whereas both ovarian carcinoids (Sporrøng et al., 1981 b) and, in particular, mucinous tumours are more difficult to preserve adequately for immunohistochemical investigations, because of their size.

The epithelium which makes up the main part of ovarian mucinous tumours is not found in the normal ovary. It is said to resemble colonic epithelium (Klemi, 1978) or that of the uterine cervix, and the prevalent hypotheses are that it arises either as a metaplasia of the ovarian germinal epithelium or as a monophyletic teratoma (Fox et al., 1964). The general appearance of the epithelial lining with its disseminated argyrophil cells of open type is rather that of an endodermally derived mucosa of foregut type, often seen in benign cystic teratomas ("dermoid cysts") of the ovary. It is becoming increasingly realized that epithelial tumours of foregut type may contain argyrophil cells, probably of endocrine nature, as shown for carcinoma of the stomach (Tahara et al., 1975), of the pancreas (Klöppel et al., 1980) and of the gall bladder (Azadeh and Parai, 1980). Whether these argyrophil cells are actual tumour cells or represent the result of concomitant metaplasia (Tahara et al., 1975) is still an open question. The present findings suggest that peptide hormone producing cells and mucin producing cells arise from a common stem cell. Some kind of histogenetic relationship between endocrine cells and mucin producing cells is suggested by previous phylogenetical studies (cf. Falkmer et al., 1973) and from observations on several kinds of human tumours and hyperplasias, such as so-called amphicrine cell proliferations and adenocarcinoid tumours of the vermiform appendix and colon (Ratzenhofer, 1977; Olsson and Ljungberg, 1980; Ratzenhofer and Auböck, 1980) and mucoid carcinoma of the breast (Capella et al., 1980).

The immunohistochemical observations made in the present study of argyrophil cells in mucinous cystadenomas and cystadenocarcinomas of the ovary indicate a spectrum of neurohormonal peptides similar to that seen in carcinoids of foregut type rather than in those of midgut or hindgut type (Alumets et al., 1980). Thus, support is obtained for the view that ovarian mucinous tumour represent monophyletic teratomas of foregut endodermal origin.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (Projects No. 4X-4499 and 12X-718), the Swedish Cancer Society (Project No. 806-5XA), the Cancer Research

Fund of Malmö General Hospital, and the Medical Faculty at the University of Lund.

REFERENCES

- Alumets, J., Falkmer, S., Håkanson, R., Ljungberg, O., Sundler, F.: Characteristic spectrum of neurohormonal peptides in endocrine tumours arising in foregut, midgut or hindgut derivatives. *Regul. Pept.* 1, S3 (1980).
- Alumets, J., Alm, P., Falkmer, S., Håkanson, R., Ljungberg, O., Mårtensson, H., Sundler, F., Tibblin, S.: Immunohistochemical evidence of peptide hormones in endocrine tumors of the rectum. *Cancer* 00, 000-000 (1981).
- Azadeh, B., Parai, S.K.: Argentaffin cells, intestinal metaplasia and antral metaplasia in carcinoma of the gallbladder. *Histopath.* 4, 653-659 (1980).
- Capella, C., Eusebi, V., Mann, B., Azzopardi, J.G.: Endocrine differentiation in mucoid carcinoma of the breast. *Histopath.* 4, 613-630, (1980).
- Cocco, A.E., Conway, S.: Zollinger-Ellison syndrome associated with ovarian mucinous cystadenocarcinoma. *New Engl.J.Med.* 293, 485-486, (1975).
- Creutzfeldt, W. (Ed.): *Gastrointestinal Hormones. Clinics in Gastroenterology* 9, 1-806 (1980).
- Dayal, Y., O'Brian, E.S., DeLellis, R.A., Wolfe, H.J.: Carcinoid tumors in gastrointestinal and extra-intestinal sites. A comparative study of polypeptide hormonal profiles. *Regul. Peptides* 1, Suppl. 1, S22 (1980).
- Efendić, S.: Somatostatin - A classical hormone. A locally active polypeptide, and a neurotransmitter. *Annals Clin.Res.* 12, 87-94, (1980).
- Falkmer, S., Emdin, S., Havu, N., Lundgren, G., Marques, M., Östberg, Y., Steiner, D.F., Thomas, N.W.: Insulin in invertebrates and cyclostomes. *Amer.Zool.* 13, 625-638 (1973).
- Fox, H., Kazzaz, B., Langley, F.A.: Argyrophil and argentaffin cells in the female genital tract and in ovarian mucinous cysts. *J.Path.Bact.* 88, 479-488 (1964).
- Friesen, S.R., Bolinger, R.E.: *Surgical endocrinology. Clinical syndromes.* Philadelphia: Lippincott Co. (1978).

- Grahame-Smith, D.G.: The carcinoid syndrome. In: DeGroot, L.J., Cahill, G.F., Odell, W.D., Martini, L., Potts, J.T., Nelson, D.H., Steinberger, E., Winegrad, A.I. (Eds): *Endocrinology* 3, New York: Grune & Stratton, pp. 1721-1732 (1979).
- Grimelius, L.: Studies of adult human pancreatic islet cells with a new silver nitrate stain. *Acta Soc.Med.Upsalien.* (1969).
- Klemi, P.J.: Pathology of mucinous ovarian cystadenomas: 1. Argyrophil and argentaffin cells and epithelial mucosubstances. *Acta Path. Microbiol.Scand., Sect. A.* 86, 465-470 (1978).
- Klemi, P.J., Nevalainen, T.J.: Pathology of mucinous ovarian cystadenomas. *Acta Path.Micrbiol.Scand., Sect. A.* 86, 471-481 (1978).
- Klöppel, G., Bommer, G., Heitz, P.U.: Immunocytochemistry of the endocrine pancreas in chronic pancreatitis and pancreatic carcinoma. In: Podolsky, S., Viswanathan, M. (Eds): *Secondary Diabetes: The Spectrum of the Diabetic Syndromes.* New York: Raven Pr. pp. 147-151, (1980).
- Long, T.T., Barton, T.K., Draffin, R., Reeves, W.J., McCarty, K.S.: Conservative management of the Zollinger-Ellison Syndrome. Ectopic gastrin production by an ovarian cystadenoma. *J.A.M.A.* 243, 1837-1839, (1980).
- McCann, S.M., Krulich, L., Negrovilar, A., Ojeda, S.R., Vijayan, E.: Regulation and function of panhibin (somatostatin). *Adv. biochem. phycopharmacol.* 22, 131-144 (1980).
- Olsson, B., Ljungberg, O.: Adenocarcinoid of the vermiform appendix. *Virchows Arch.A.Path.Anat.Histol.* 386, 201-210 (1980).
- Ratzenhofer, M.: Ueber enterale Hyperplasien und Geschwülste der disseminierten endokrinen (parakrinen) Hellen Zellen Feyrters unter Berücksichtigung amphikriner Zellwucherungen. *Verb.Dtsch. Ges.Path.* 61, 7-24 (1977).
- Ratzenhofer, M., Auböck, L.: The amphotrine (endo-exocrine) cells in the human gut, with a short reference to amphotrine neoplasias. *Acta Morphol.Acad.Sci.Hung.* 28, 37-58, (1980).
- Schmid, K.O.: Ueber disseminierte endokrine (Feyrter) Zellen in Mucinkystomen des Ovars. *Verh.Dtsch.Ges.Path.* 61, 143-148 (1977).
- Scully, R.E.: Tumors of the ovary and maldeveloped gonads. In: *Atlas of Tumor Pathology.* 2nd ser., Fasc. 16, Washington, D.C.: Armed Forces Inst.Path., p. 314, (1980).
- Serov, S.F., Scully, R.E.: Histological typing of ovarian tumours. *W.H.O. Internat.Histol.Classif. Tum.* 9, 1-123, (1973).

- Solcia, E.: Endocrine cells of the gastrointestinal tract and related tumors. *Pathobiol. Annual* 9, 163-204 (1979).
- Sporrong, B., Falkmer, S., Robboy, S.J., Alumets, J., Håkanson, R., Ljungberg, O., Sundler, F.: Evidence for neurohormonal peptides in ovarian carcinoids. A preliminary report of immunohistochemical findings. *U.C.L.A. Forum Med. Sci.* 23, 257-265 (1981a).
- Sporrong, B., Falkmer, S., Robboy, S.J., Alumets, J., Håkanson, R., Ljungberg, O., Sundler, F.: Neurohormonal peptides in ovarian carcinoids. An immunohistochemical study of eighty-one primary carcinoids and of intraovarian metastases from six mid-gut carcinoids. *Cancer* 00, 000-000 (1981b).
- Sternberger, L.A.: *Immunocytochemistry* (Ed.2) New York: John Wiley & Sons, pp. 1-354 (1979).
- Tahara, E., Haizuka, S., Kodama, T., Yamada, A.: The relationship of gastrointestinal endocrine cells to gastric epithelial changes with special reference to gastric cancer. *Acta Path. Jap.* 25, 161-177, (1975).

TABLE 1. Specification of the GEP neurohormonal peptide antisera used and their source of origin and working dilutions.

Antisera raised against	Working IF *)	Dilution PAP **)	Code	Source
Pure bovine insulin	1/80	1/5120	LAI	L.G. Heding, Novo Res.Inst., Bagsvaerd, Denmark
Synthetic ovine somatostatin (SOM)		1/2560	19578	M.P. Dubois, Station Physiol.Reprod., INRA, Nouzilly, France
Pure porcine glucagon		1/640	7811	J.E. Thorell, Dept.Nucl.Med., Malmö Gen. Hospital, Malmö, Sweden
Pure bovine pancreatic polypeptide (PP)	1/320	1/2560	7823	J.E. Thorell, Dept.Nucl.Med., Malmö Gen. Hospital, Malmö, Sweden
Pure porcine secretin	1/80	1/2560	5585	O.B. Schaffalitzky de Muckadell, Bispebjerg Hospital, Copenhagen, Denmark
Pure porcine vasoactive intestinal polypeptide (VIP)		1/5120	5603	J.Fahrenkrug, Dept.Clin.Chem., Copenhagen County Hospital, Glostrup, Denmark
		1/5120	7852	J.E. Thorell, Dept.Nucl.Med., Malmö Gen.Hospital, Malmö, Sweden
Synthetic human gastrin 2-17		1/5120	4562	J.F. Rehfeld, Dept.Med.Biochem., University Aarhus, Aarhus, Denmark
Synthetic bovine substance P (SP)	1/20		K 16	G.Nilsson, Dept.Pharmacol., Karolinska Inst., Stockholm, Sweden
Synthetic bovine neurotensin (NT)	1/80	1/640	SP-8	P.C. Emson, Med.Res.Council, Cambridge, England
			HC-8	R.E. Carraway, Lab.Hum.Reprod., Dept.Physiol., Harvard Med.Sch., Boston, Ma, USA
Synthetic leu- or met-enkephalin (Enk)	1/240	1/240	Leu-enk	R.J.Miller & K.J. Chang, Burroughs Wellcome Res. Lab., Triangle Park, NC, USA
		1/640	Met-enk	
Synthetic human β -endorphin (β -End) (β -lipotropin 61-91)		1/640	7763	J.E. Thorell, Dept.Nucl.Med., Malmö Gen.Hospital, Malmö, Sweden
Synthetic porcine motilin		1/640	MBR-1105	N. Yanaihara, Shizuoka Coll.Pharm., Shizuoka, Japan
Synthetic human calcitonin (CT)		1/160	AS292/5	I. MacIntyre, Endocr.Unit, Royal Postgrad.Med. Sch., London, England
Pure porcine adrenocorticotrophic hormone (ACTH)	1/80	1/640	No. 1	Own

*) IF: Immunofluorescence **) PAP: Peroxidase-Anti-Peroxidase procedure

COMMENTS TO TABLE 1:

The antisera were inactivated by the respective antigen. The antigens were obtained from the following sources:

Insulin and glucagon were both kindly donated by Dr L.G. Heding, Novo Research Institute, Bagsvaerd, Denmark.

Somatostatin, substance P, enkephalin, β -endorphin, and calcitonin, were all purchased from Peninsula Inc., San Carlos, Calif., USA.

BPP was generously supplied by Dr R.E. Chance, Lilly Res. Lab., Indianapolis, Indiana, USA.

Secretin, VIP, and motilin, were kindly provided by Prof. V. Mutt, Dept. of Biochem. II, Karol. Inst., Stockholm, Sweden.

Gastrin-17 was donated by Prof. J.F. Rehfeld, Dept. of Med. Biochem., University of Aarhus, Aarhus, Denmark.

Neurotensin was bought from U.C.B., Brussels, Belgium.

ACTH was purchased from Ferring Inc., Malmö, Sweden.

Table 2. Relative incidence of argyrophil epithelial cells in the parenchyma of ovarian cystadenomas and cystadenocarcinomas (Grimelius' procedure).

	Total number	0	+	++	+++	% *)
Benign Cystadenoma	144	118	17	8	1	18
Cystadenoma of Borderline Type	33	22	6	2	3	33
Cystadenocarci- noma	64	30	4	16	14	53
Total	241	170	27	26	18	29

+ : Occasional argyrophil cells

++ : Moderate numbers of argyrophil cells

+++ : Numerous argyrophil cells

*) Per cent of tumours with at least "+" incidence of argyrophil tumour cells.

TABLE 3. Follow up and survey of some histopathological and immunohistochemical data from those 12 patients with ovarian cystadenomas and cystadenocarcinomas where cells immunoreactive with antisera raised against GEP neurohormonal peptides were detected.

Case no.	Patient Age in years at op.	Histo-pathol. tumour type a)	Relative incidence of argyrophil cells b)	Relative incidence ^{c)} of cells immunoreactive with antisera raised against SOM ^{d)} GLUC ^{d)} GAST/CCK ^{d)} ENK ^{d)} NT ^{d)}	Follow up
1	52	12864/76 IIb	++	+	No symptoms after 4 years
2	45	15568/78 IIb	+++	++	No symptoms after 2 years
3	39	581/70 IIC	+++	+	No symptoms after 11 years
4	63	5954/70 IIC	+	+	No follow up
5	66	19557/71 IIC	+++	+	No symptoms after 2 years. No further follow up.
6	51	13472/72 IIC	++	++	No symptoms after 8 years
7	54	7652/74 IIC	+++	+	No symptoms after 6 years
8	55	7795/75 IIC	+++	+	No symptoms after 5 years
9	54	14025/75 IIC	++	++	Dead after 4 years with pulmonary metastases
10	21	503/77 IIC	+++	+	No symptoms after 3 years
11	65	1219/77 IIC	+++	++	No symptoms after 3 years
12	48	11088/78 IIC	+++	+	No symptoms after 2 years

a) IIb: Mucinous cystadenomas of borderline malignancy.

b) IIC: Mucinous cystadenocarcinomas.

Same semiquantitative grading as in Table 2.

c) + : Occasional immunoreactive epithelial cells in the tumour parenchyma

++ : Moderate numbers of immunoreactive epithelial cells in the tumour parenchyma

d) SOM: Somatostatin
GLUC: Glucagon
GAST/CCK: Gastrin/Cholecystokinin
ENK: Enkephalin
NT: Neurotensin

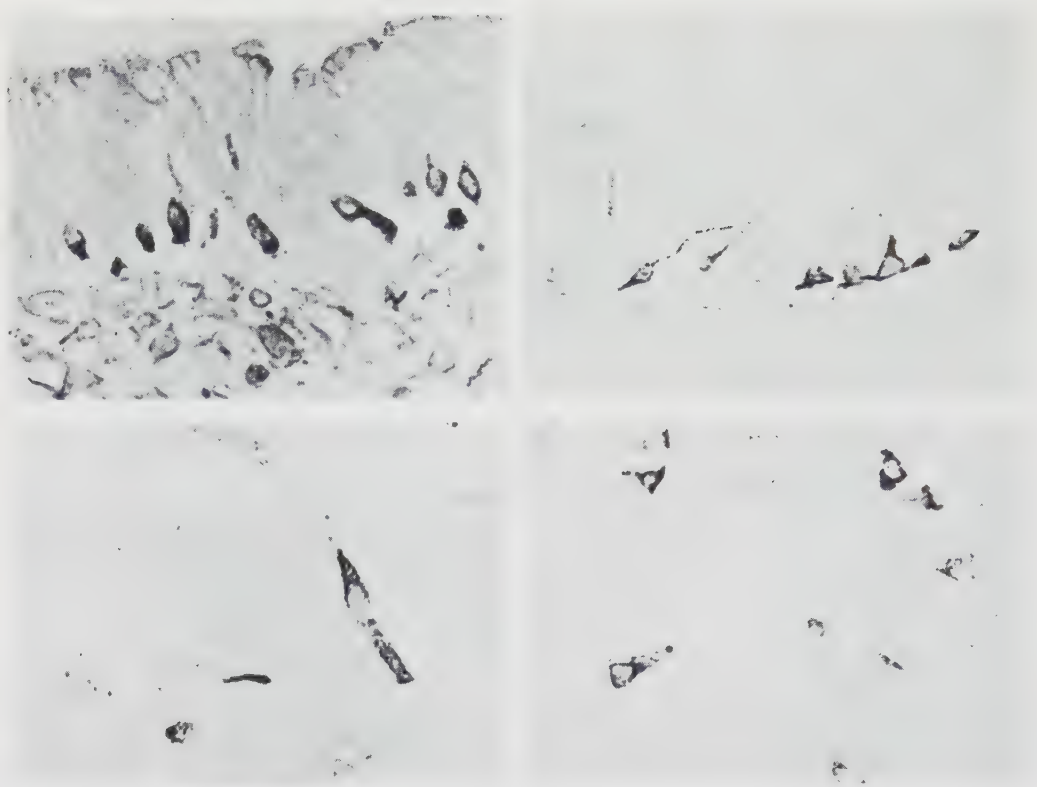


Fig. 1: Medium-power photomicrographs of four ovarian mucinous cystadenocarcinomas, showing the widely varying structure of the argyrophil cells (black) in the tumour epithelium. Grimelius' silver nitrate procedure. X 200

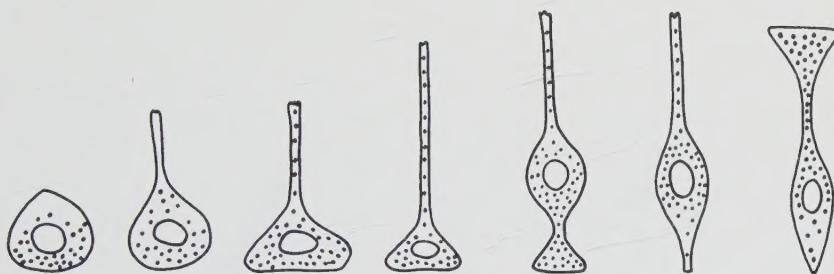


Fig. 2: Schematic outline of the spectrum of argyrophil cell types seen in the tumour epithelium of ovarian mucinous cystadenomas and cystadenocarcinomas. All cells stand on the basal membrane of the epithelium; some are of closed type (left), *i.e.*, they do not seem to reach the luminal surface; others are of open type, being equipped with apical processes of varying shape (middle and right), extending all the way from the basal membrane to the microvillous border at the lumen.

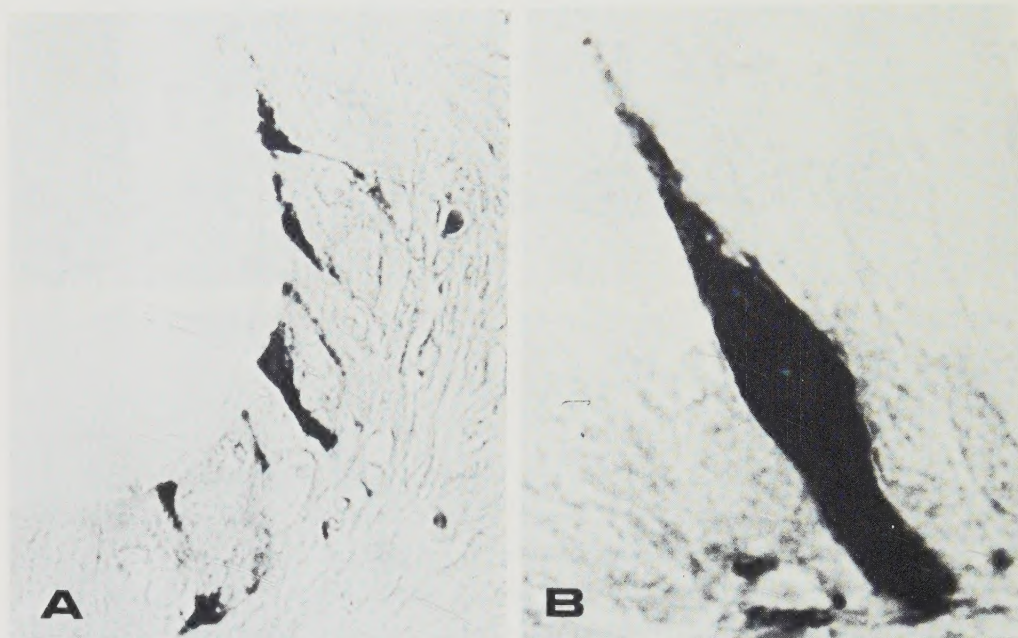


Fig. 3: Medium (A) and high-power (B) photomicrographs of two ovarian mucinous cystadenocarcinomas, showing cells in the tumour epithelium displaying immunoreactivity with antisera against (met)enkephalin (A) and gastrin/CCK (B), respectively.

The general structure of the immunoreactive cells conforms to that of the argyrophil cells (Figs. 1 and 2).

PAP procedure. X 800 (A) and 2,000 (B).

